

Studies on
HIGH DENSITY LIPOPROTEINS
with special reference to characterization of HDL changes
in chronic alcoholism and biliary obstruction

by
Rolf Ekman



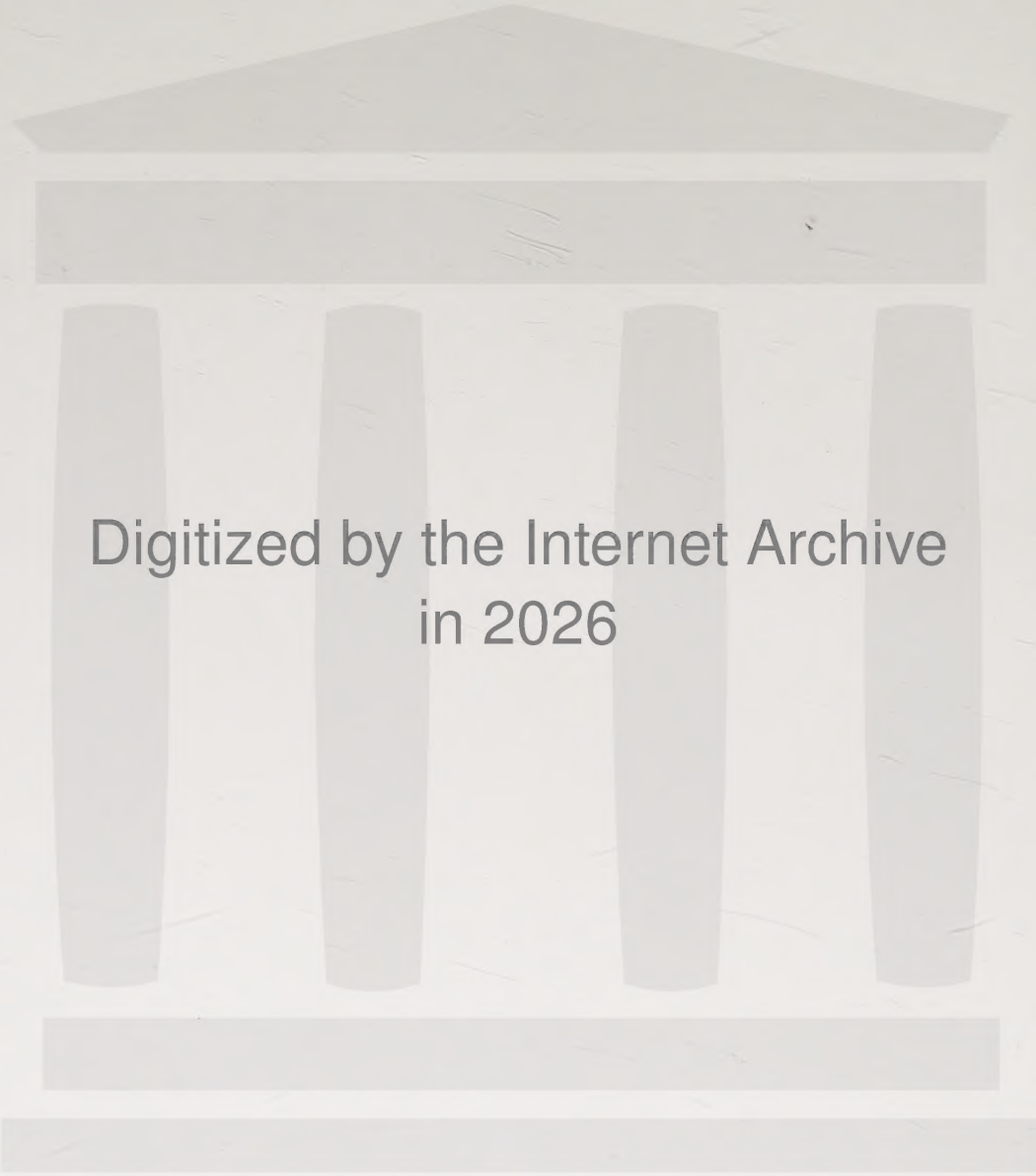
Lund 1978

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av

Rolf Ekman
med lic, Sm

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From the Department of Clinical Chemistry, University Hospital,
S-221 85 Lund, Sweden

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This thesis is based on the following papers referred to in the text by their Roman numerals:

- I Separation of plasma lipoproteins by zonal ultracentrifugation
Submitted for publication
In collaboration with B. Danielsson and B.G. Johansson
- II Immunochemical studies of lipoproteins in cholestasis
Scand. J. Immunol. 4, Suppl. 2 (1975) 13-18
In collaboration with B.G. Johansson and B.-G. Petersson
- III Characterization of high density lipoproteins in human
cholestasis
Scand. J. Clin. Lab. Invest. 37 (1977) 587-597
In collaboration with B. Arnesjö, B. Danielsson,
B.G. Johansson and B.G. Petersson
- IV Isolation and partial characterization of lipoprotein E from
patients with long-standing biliary obstruction
Submitted for publication
In collaboration with B. Danielsson, B.G. Johansson,
P. Nilsson-Ehle and B.G. Petersson
- V Changes in plasma high density lipoproteins in chronic male
alcoholics during and after abuse
Scand. J. Clin. Lab. Invest. 38 (1978) in press
In collaboration with B. Danielsson, G. Fex, B.G. Johansson,
H. Kristensson, P. Nilsson-Ehle and J. Wadstein
- VI Isolation of a high density lipoprotein with high contents of
arginine-rich apoprotein (apo E) from rat plasma
FEBS Lett. 86 (1978) 299-302
In collaboration with B. Danielsson, B.G. Johansson,
P. Nilsson-Ehle and B.G. Petersson



"Lustigt vad man för
en smula honung gör
surr, surr, surr,
jag undrar just varför"

(Nalle Puh)

ABBREVIATIONS

Apo A-I	apolipoprotein A-I
Apo A-II	apolipoprotein A-II
Apo B	apolipoprotein B
Apo C-I	apolipoprotein C-I
Apo C-II	apolipoprotein C-II
Apo C-III	apolipoprotein C-III
Apo D	apolipoprotein D
Apo E	apolipoprotein E
EIA	electroimmuno assay
HDL	high density lipoproteins
HDL ₁ -C	cholestatic high density lipoprotein with d = 1.08 kg/l
IDL	intermediate density lipoproteins
LpA	lipoprotein A (lipoprotein family containing apo A-I and apo A-II)
LpB	lipoprotein B (lipoprotein family containing apo B)
LpC	lipoprotein C (containing apo C-I, apo C-II, and apo C-III)
LpD	lipoprotein D
LpE	lipoprotein E
LpX	lipoprotein X
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoproteins
PAGE	polyacrylamide gel electrophoresis
RCF	relative centrifugal field
SDS	sodium dodecylsulphate
VHDL	very high density lipoproteins
VLDL	very low density lipoproteins

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INTRODUCTION

Plasma lipoproteins

The transport in plasma of non-polar lipids (triglycerides and cholesteryl esters) and polar lipids (phospholipids and unesterified cholesterol) is achieved by the formation of non-covalent lipid-protein complexes called plasma lipoproteins. Studies with physical, chemical, and electron microscopic techniques suggest that these lipoproteins have the same basic structure and have demonstrated that they consist of a hydrophobic core of triglyceride and/or cholesteryl esters surrounded by an amphiphilic monolayer composed of phospholipids, cholesterol, and proteins called apolipoproteins (1-7). The plasma lipoproteins can be regarded as a spectrum of heterogeneous macromolecules, which vary widely in size and composition. Classification of the plasma lipoproteins is generally based on their different physicochemical properties, such as charge (electrophoresis) (8, 9) or hydrated density (ultracentrifugation) (10, 11) or apolipoprotein composition (12). In Table I the composition and some properties of various plasma lipoproteins are summarized.

The apolipoproteins or apoproteins, which have an amphipathic character, are localized in the polar surface coat (7) of the plasma lipoproteins. Eight apoproteins, which are presented in Table II, using the nomenclature of Alaupovic (12), are generally recognized in human lipoproteins. This nomenclature is also related to the lipoprotein family concept suggested by Alaupovic et al. (12), which postulates the existence of lipoprotein families, i.e. LpA, LpB, LpC, LpD, and LpE. The amino acid composition of the eight apoproteins is known, while the sequence is as yet known for five (apo A-I, A-II, C-I, C-II, C-III) (13). For apo B, the molecular weight determined by Tanford's group is given (16). The exact molecular weight of apo E is still unknown, but is obviously in the vicinity of 35 000 (17-19).

It has been suggested that some of the apoproteins are important for maintaining lipoprotein structure, e.g. apo B and possibly apo A-II (20), while others seem to have a function in the regulation of the metabolic processes of lipoprotein transformation and degradation. For example, apo C-II is the activator of lipoprotein lipase (21, 22) and apo A-I is the main activator of the enzyme responsible for cholesterol esterification in plasma lecithin:cholesterol acyltransferase (LCAT) (23). Apo E might be of importance in the cellular uptake of certain lipoproteins (24). In addition to the well established apoproteins described, several others have been

TABLE I

Properties, chemical composition and mean concentration of plasma lipoproteins in normal subjects.

Data mainly from references 13 and 14.

Properties	Chylo- microns	VLDL	IDL (LDL ₁)	LDL	HDL ₂	HDL ₃	VHDL ₁
Electrophoretic mobility (agarose)	origin	pre-β	pre-β-β	β	α	α	α
Density (kg/l)	< 0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.125	1.125-1.21	1.21-1.25
Particle weight	> 0.4·10 ⁹	5-10·10 ⁶	3.9-4.8·10 ⁶	2.7·10 ⁶	3.9·10 ⁵	1.8·10 ⁵	-
Diameter (nm)	> 75	25-75	22-24	20-23	6-14	4-10	-
<u>Composition (%)</u>							
Protein	2	8	-	21	41	55	63
Triglyceride	84	50	-	11	5	4	5
Cholesterol, unesterified	2	7	-	8	5	3	1
Cholesteryl ester	5	12	-	37	16	12	3
Phospholipid	7	18	-	22	30	23	28
Lipoprotein levels (g/l)							
males (35-55 years)	b)	1.59±1.16	-	3.69±0.81	0.53±0.48	2.22±0.31	-
females (35-55 yrs)	b)	0.56±0.6	-	3.03±0.36	1.72±0.81	2.64±0.59	-
Biological half-life ^{a)}	< 1 hr	2-4 hrs	mins ?	2.5 days	5 days	5 days	-

a) Ref. 15.

b) Variable; dependent on fat ingestion.

TABLE II
Characteristics and occurrence of the apoproteins of
human plasma lipoproteins. Data mainly from ref. 13.

Apoprotein	Molecular weight	Number of amino acid residues	Lipoprotein family	Lipoprotein classes
A-I	28 331	293	LpA	HDL (VLDL)
A-II	2 x 8 690	2 x 77		HDL (VLDL)
B	(255 000) ?	-	LpB	LDL, VLDL
C-I	6 630	57	LpC	VLDL, HDL
C-II	8 837	78		VLDL, HDL
C-III	8 764	79		VLDL, HDL
D	22 100	-	LpD	HDL
E	(33 000; 39 000) ?	-	LpE	VLDL (HDL)

isolated, inter alia a protein rich in serine and glycine which is present in small amounts in HDL (25).

The traditional classifications of plasma lipoproteins are based mainly on physicochemical properties like hydrated density and electrophoretic mobility. From a functional point of view the lipoproteins can be divided into two separate but interacting systems, namely VLDL-IDL-LDL and HDL.

The function of the VLDL-LDL system is to transport triglyceride from the liver and intestine to muscle, adipose and other tissues. Hepatogenic VLDL and chylomicrons derived from the gut are large spherical triglyceride-rich particles, which vary greatly in size and chemical composition (Table I). In VLDL and chylomicrons the major proteins are apo B and apo C, representing more than two thirds of the total protein in VLDL. VLDL and chylomicrons are metabolized by sequential hydrolysis of the triglyceride component ultimately resulting in the formation of LDL particles (15). IDL seem to be short-lived intermediate particles in this sequence (26). It has been demonstrated that the initial hydrolysis of lipoprotein triglyceride is catabolized by the enzyme lipoprotein lipase (27),

whereas the details of the subsequent degradation from IDL to LDL are less well known. LDL are most probably eliminated in tissues other than the liver by cellular uptake mediated via specific receptors (28).

Normal human HDL

The HDL class consists of comparatively small spherical particles with diameters varying from 4 to 14 nm with a core of mainly cholesteryl esters. The HDL particles have α -mobility on electrophoresis; the designation α -lipoprotein is sometimes used as a synonym for HDL.

The heterogeneous density distribution of HDL in the analytical ultracentrifuge has formed the basis for the separation of HDL into subclasses. Two major subclasses have been found in human plasma, HDL₂ and HDL₃ (10), which have identical but proportionally different lipid and protein constituents. The minor HDL₁ subclass is poorly defined. According to Albers et al. (29) the material in the density region roughly corresponding to HDL₁ ($d = 1.060-1.075$) contains lipoproteins of both HDL and LDL nature. It should also be mentioned that lipoproteins of a higher density than that of HDL have been isolated. This VHDL fraction seems to consist mainly of apo A-I-phospholipid complexes and trace amounts of albumin (30). Recently an albumin-apo A-I complex has been isolated by affinity chromatography followed by preparative electrophoresis. This complex is probably present in vivo (31).

Human HDL contain about 50% lipids (Table I). The dominating lipids are phospholipids and cholesterol, two thirds of the latter being present in esterified form. The main fatty acid in the cholesteryl esters is linoleic acid (14). Only small amounts of triglycerides are found in HDL. The main apoproteins of HDL are apo A-I and apo A-II comprising about 90% of the protein moiety. Apoproteins C are found in small amounts in the entire HDL density region, while the presence of apo D seems to be confined to HDL₃ (32). Small amounts of apo E have also been found in normal HDL (19).

The HDL levels in healthy individuals show variations with age and sex. Women in fertile age have somewhat higher concentrations of HDL than men in the corresponding age (33); this is due to higher levels of HDL₂ in females. The HDL₂ levels fluctuate with the menstrual cycle, with a 50% increase at ovulation (34). On the other hand, high HDL levels in pregnancy are evidently due to an increase of HDL₃ (33). Androgens, however, have been reported to decrease the HDL levels (35, 36). Other factors than age and sex must also be taken into account in the interpreta-

tion of the results of HDL determinations. For example, an inverse relationship between HDL and VLDL concentrations has been observed in several studies (37-39).

Methodological considerations are also important in the interpretation of the results of HDL determinations. Several methods, e.g. ultracentrifugation, electrophoresis and determinations of HDL constituents like cholesterol or apo A-I, have been used for the quantitation of HDL. It is obvious that these various methods will give different results in conditions associated with qualitative changes in HDL or with changes in the proportions of HDL subclasses.

Present knowledge of the metabolism of HDL is limited. It seems reasonable to distinguish between three different metabolic phases: (1) synthesis and secretion of newly synthesized lipoprotein from liver or intestine, (2) transformation of this nascent lipoprotein in plasma, and (3) cellular uptake and elimination of lipoprotein. In rats, lipoprotein particles containing apo A-I seem to be synthesized and secreted from both liver (40) and intestine (41), while no information is available on the synthesis of apo A-II. Little information on the synthesis and secretion of human nascent HDL is available. The nascent HDL particles are assumed to be transformed to normal plasma HDL by the action of the transacylating enzyme LCAT, with the formation of non-polar cholesteryl esters. These esters accumulate in the core of the lipoprotein, leading to a change from disc-shaped to spherical particles. The transformation is accompanied by transfer of protein and lipid material between HDL and other plasma lipoprotein classes. Extensive studies on lipid and lipoprotein metabolism in patients with familial LCAT deficiency have been extremely useful in elucidating the role of LCAT in the formation of normal plasma HDL (42).

Details concerning the fate of plasma HDL are scanty. The half-life of human HDL labelled with radioactive iodine is approximately 5 days (43). The liver and kidney, and to a lesser extent the reticuloendothelial system and small intestine have been suggested as organs responsible for HDL catabolism (44), but the precise site(s) and mechanisms for these events are so far unknown. Recent studies have shown that rat hepatocytes were able to take up and degrade rat HDL (45, 46). Uptake has also been demonstrated in non-parenchymal liver cells, probably mediated by other mechanisms than that in the hepatocytes (46).

The function of HDL remains speculative. It is suggested from available information that the LCAT reaction promotes a net transport of unesterified cholesterol from cell membranes to HDL (47). Since the liver

may be the main site of HDL catabolism, an important function of HDL could be to transport cholesterol to the liver for excretion into bile. Recent studies have suggested that rat adrenal tissue takes up HDL cholesterol by an HDL specific hormonally responsive mechanism and that apo A-I and apo A-II have a significant function in the uptake process (48).

HDL have also been suggested to have an important function in the metabolism of chylomicrons and VLDL, serving as a reservoir for apo C. These apoproteins, including the lipoprotein lipase activating apo C-II, are transferred to newly secreted chylomicrons and VLDL. During the degradation of the triglyceride core of chylomicrons and VLDL by the action of lipoprotein lipase, excess surface material (apo C, unesterified cholesterol and phospholipid) is transferred to HDL (15). The amphiphilic surface lipids are converted to non-polar core material in the HDL particle by the action of LCAT and the cholesteryl ester formed seems to be transferred back to the VLDL-IDL-LDL system (49).

Recently, HDL have attracted considerable interest from a clinical point of view. A growing number of observations indicate that HDL may be a factor preventing progress of atherosclerotic lesions, an effect possibly related to the function of HDL in the transport of cholesterol from peripheral tissues to the liver discussed above. Epidemiological studies have demonstrated lower HDL levels in patients with a history of myocardial infarction than in matched controls (50). The prolonged life expectancy found in the recently discovered familial condition, hyperalphalipoproteinemia (51), emphasizes the possible beneficial effect of HDL. Furthermore, *in vitro* experiments indicate that HDL may play a role in the transport of cholesterol out of atherosclerotic lesions (52). It is not known at present if the entire HDL is involved in this possible retardation of atherogenesis or if only special subfractions are operative.

HDL in disease

A variety of conditions are accompanied by changes in HDL concentrations (Table III). A few disorders with very low levels of HDL are due to primary derangement in HDL metabolism, i. e. Tangier disease (53) and familial LCAT deficiency (54). Severe reductions in HDL have also been reported in abetalipoproteinemia (55).

Patients with familial hyperlipoproteinemias frequently have low HDL concentrations with one exception, familial broad β disease type III, which shows no remarkable changes in HDL concentration (56).

Decreased levels of HDL also seem to be a common finding in con-

TABLE III
HDL levels in various conditions

Low levels	High levels
Androgen influence	Estrogen influence
Tangier disease	Familial hyperalphalipoproteinemia
Familial LCAT deficiency	Alcohol overconsumption
Abetalipoproteinemia	Exposition to pesticide
Familial hyperlipoproteinemias	
Diseases with inflammatory response	
Renal disease	
Liver disease	

ditions with an inflammatory reaction, including such common diseases as myocardial infarction and infections (57) which are revealed by characteristic changes of the plasma protein profile. The cause of the observed decrease in HDL concentrations in these cases is speculative, but detailed studies in the postoperative period after cholecystectomy (58) and after myocardial infarction (59) have shown that the decrease of HDL is parallel to that found for albumin, indicating the possibility that the same mechanisms are responsible for the HDL changes as for albumin, i.e. a shift from intravascular to extravascular space in combination with increased catabolism (60, 61).

The HDL levels are often subnormal in renal disease (33). The demonstration of HDL in urine from nephrotic patients (62, 63) suggests that the low plasma levels of HDL are at least partly explained by urinary losses of HDL having roughly the same particle size as immunoglobulin G, which is also lost in the urine in nephrosis.

Studies of plasma from uremic patients using lipoprotein electrophoresis revealed low levels of serum α -lipoprotein, especially when the patients were maintained by chronic dialysis (64). The α -lipoprotein concentration rose to normal or near normal when renal function was restored two or three weeks after transplantation. In contrast to this observation recent reports indicate that the HDL cholesterol remains subnormal after successful transplantation (65, 66), suggesting that other factors than

kidney function are responsible for the decreased HDL in the post-transplant state.

Several investigators, using different techniques, have noted low values of HDL in liver-biliary diseases, for example in acute infectious hepatitis (67-69), toxic hepatitis (70), primary biliary cirrhosis (67, 71), and obstructive jaundice of different etiology (67, 68, 71, 72). In a few cases of obstructive jaundice the HDL levels have been reported to be very low or even absent (68). The reduction of HDL found in liver-biliary diseases is often associated with marked qualitative changes of the lipid composition of HDL, e.g. increased contents of unesterified cholesterol and phospholipids (67, 68). Subsequent studies (73-76) have correlated LCAT activity with various liver diseases and demonstrated marked reduction in the activity of LCAT.

It has further been suggested that apo A of HDL obtained from patients with disturbed liver function undergo changes, which can be revealed by dissociation of these apoproteins in immunoelectrophoresis (77).

Cholestatic HDL and HDL fractions isolated by differential ultracentrifugation from patients with biliary obstruction have been studied by electron microscopy and found to contain mainly disc-shaped particles, completely different from normal HDL (78, 79) but similar to lipoprotein particles observed in HDL from patients with familial LCAT deficiency (79, 80).

So far only a few conditions have been reported to be associated with increased levels of HDL. A familial form of hyperalphalipoproteinemia occurring in apparently healthy persons, was recently described (51, 81). These individuals are usually discovered incidentally and have about twice the normal content of HDL. The increase in HDL comprises both HDL₂ and HDL₃ which have apparently normal lipid and apoprotein composition (82, 83).

In our experience, the overwhelming majority of cases with elevated HDL concentrations can be linked to either estrogen influence or alcohol consumption. It is well known that alcohol consumption gives rise to increase of the plasma triglyceride (VLDL) levels (84-86), which may sometimes be very pronounced after alcohol abuse. Also other lipoproteins, e.g. HDL, may be affected by alcohol intake, as first demonstrated in chronic alcoholics, which were found to have increased HDL levels, often in combination with an abnormal distribution in crossed immunoelectrophoresis (87). Several reports have followed this primary observation and

confirmed a relationship between ethanol intake and elevated HDL concentrations (88-91). It has been reported that exposure to chlorinated pesticides, mainly lindane, may also raise the α -lipoprotein levels (92). Cessation of the exposure to the pesticide caused a decrease in α -lipoprotein in all subjects except one (93).

Present investigations

Despite the growing interest in high density lipoproteins in the light of their unequivocal importance for the transport and/or metabolism of plasma lipids, e.g. cholesterol and triglycerides, as well as their suggested role as a factor protecting against atherosclerotic cardiovascular disease, our knowledge of HDL metabolism and its changes in disease is rather limited. The aim of the present work is to contribute to the characterization of HDL changes in two patient categories with different types of HDL alterations, i.e. chronic alcoholics after recent debauches and patients with long-standing obstructive jaundice.

The main analytical tools used in this study were zonal ultracentrifugation and modern immunoelectrophoretic techniques - electroimmunoassay and crossed immunoelectrophoresis.

MATERIALS

Patients

The following patient categories were included in this study:

1. Male alcoholics. The 38 patients described in paper V were all γ -alcoholics according to Jellinek's classification system (94) who were admitted to the Alcohol Clinic after a recent debauché lasting for at least four weeks.
2. Patients with obstructive jaundice. Fourteen patients with biliary obstruction due to malignant tumours or gall stones were studied (III, IV). In addition, two patients with alcoholic liver disease associated with severe jaundice were included in paper IV.
3. Patients with inflammatory conditions. This group consisted of 10 patients with distinct signs of "inflammatory reaction" in the plasma protein profile. None of these patients had signs of biliary obstruction or liver disease (III).
4. Patients with liver cirrhosis. Three patients with histologically verified diagnosis but in an anicteric phase were used. All three patients

had plasma protein profiles indicative of liver dysfunction (95).

5. Healthy individuals. Apparently healthy individuals (15 males and 4 women), 25-51 years of age, were used as controls.

Blood samples were drawn after 12-14 hours' overnight fast from the patients and the healthy volunteers presented above.

Animals

In paper VI, male Sprague-Dawley rats weighing 250-300 g were used. The rats were fed a commercial pelleted laboratory chow (Astra-Ewos Old Brood Stock Maintenance Feed for Rats and Mice). Blood samples were drawn by terminal aorta puncture under light ether anaesthesia. The animals were fasted 16 hours before sampling.

Preparation of lipoproteins and apolipoproteins

In addition to the zonal ultracentrifugation procedures described in detail below, differential ultracentrifugation (96) was used for the preparation of the main lipoprotein density classes (II). Lipoprotein X was prepared by polyanion precipitation followed by ultracentrifugation (II).

Apo A-I and apo A-II were prepared as described by Scanu et al. (97) and apo C-I as described by Shore and Shore (98). Apo E was made from the abnormal HDL₁ fraction of cholestatic plasma, with the procedure used by Curry et al. (19). The purity of the apoprotein preparations was tested by polyacrylamide gel electrophoresis (PAGE) in 6 M urea and sodium dodecylsulphate (SDS).

Antiserum production

Antisera against lipoproteins or apolipoproteins were raised in rabbits by intramuscular injections of 0.2-0.5 mg of the antigens emulsified with Freund's adjuvant (II, III, IV).

Antisera against whole lipoproteins (anti-LDL, anti-HDL, and anti-LpX) were absorbed to render them specific. Anti-LDL was obtained by absorption with the infranatant fraction of normal plasma centrifuged at 1.063 kg/l. Anti-HDL was absorbed with the infranatant fraction obtained by ultracentrifugation of normal plasma at a density of 1.25 kg/l and with pure normal LDL. Anti-LpX was absorbed with normal LDL and the 1.063 kg/l infranatant. After absorption the antisera were characterized by crossed immunoelectrophoresis against plasma, purified lipoproteins and apoproteins. In some control experiments the very sensitive crossed immunoelectrophoresis with intermediate gels (99) was used as a purity

control of the antiserum preparations. The anti-LDL contained only antibodies against LpB. The antibody composition of the anti-HDL used was not characterized in detail. It was shown, however, to contain anti-apo A-I and anti-apo A-II and gave no reaction with VLDL, LDL or plasma proteins.

The antisera against apolipoproteins (apo A-I, A-II, C and E) were absorbed to render them monospecific with pure apoprotein preparations and with the ultracentrifugation fraction of normal plasma obtained at $d = 1.25 \text{ kg/l}$. Anti-apo A-I and anti-apo C were absorbed only with the infranatant fraction. Anti-apo A-II and anti-apo E were absorbed with apo A-I and the infranatant fraction.

The immunoglobulin (Ig) fraction was prepared from the antiserum with the octanoic acid precipitation technique described by Steinbuch and Audran (100). The use of the Ig fraction of the antiserum is preferred since the washing procedure in the gel immunoprecipitation technique is greatly facilitated and since excess antigen, which may be present in the antiserum after the absorption, is most often removed by the fractionation procedure.

METHODS

Immunochemical methods

Electroimmuno assay (EIA) was performed as described by Laurell (101). The runs were usually performed at 3 V/cm for 18-24 hours. As standard was used pooled plasma from blood donors, which was kept at $+4^{\circ}\text{C}$ for a maximum time of four weeks. Delipidated plasma samples were used in some experiments and were obtained by treatment of plasma with a mixture of diisopropylether:n-butanol, 60:40 v/v (102).

Crossed immunoelectrophoresis (103) was carried out as described by Ganrot (104). In experiments using intermediate antibody-containing gels we used the procedure described by Axelsen (99), which is based on a modified technique for quantitative crossed immunoelectrophoresis developed by Clarke and Freeman (105).

Immunosorbent chromatography was performed on columns containing Sepharose to which antibodies had been linked. The preparation of pure antibodies, conjugation procedure and performance of experiments are described in detail in paper IV.

Comments on the immunochemical methods

Electroimmuno assay was used mainly for two purposes in the present work, i.e. in quantitation of HDL (α -lipoprotein) and for the estimation of apoprotein distribution in fractions obtained by zonal ultracentrifugation or gel chromatography.

The results of the immunochemical determinations of HDL must be interpreted with great caution since several prerequisites must be fulfilled to obtain accurate results. First, the antiserum must not react with lipoproteins belonging to other density classes than HDL. This demand was met by the immunoabsorption of anti-HDL to render it specific for HDL. The use of anti-apo A-I was also justified for HDL determinations since only trace amounts of material reactive with this antiserum outside the HDL region of the zonal ultracentrifuge patterns were found.

Second, the immunoprecipitability and the electrophoretic mobility of the standards and samples should be as similar as possible. This is a minor problem in studies of patients with a normal HDL pattern in crossed immunoelectrophoresis, e.g. alcoholics (V) or patients with inflammatory conditions (III). In such cases it was also found that determinations of HDL with anti-HDL or apo A-I gave essentially the same results (unpublished data).

On the other hand it might be suspected that determinations of HDL by EIA in plasma from patients with obstructive jaundice (III) would give less accurate results with regard to the marked alterations seen in crossed immunoelectrophoresis and also revealed in EIA by the change of immunoprecipitate morphology and stainability with Sudan Black (II). These data indicate qualitative changes of HDL, and the immunochemical levels of HDL (α -lipoprotein) given in paper III should therefore not be interpreted as exact levels of HDL in cholestasis but merely be taken as an indication of decreased HDL concentrations.

In the present study a plasma pool from male blood donors was used as standard and the levels of HDL were generally expressed in per cent of the mean level for the reference group used (III, V). Another approach would be to determine the plasma content of apo A-I and/or apo A-II, which occur mainly in HDL. Accurate measurements of these apoproteins would seem to demand the use of delipidated samples with the pure apoprotein as standard (106). The simple delipidation procedure used in paper IV would greatly facilitate this approach. Determinations of the apo A-I content of delipidated plasma from a few patients with long-standing obstructive jaundice showed very low levels of apo A-I (around 10% of delipidated

samples of the normal plasma used as standard (cf. paper IV). The values were still expressed relative to the mean value of the reference group, since pure apo A-I samples run simultaneously in EIA gave immunoprecipitates of another shape than the plasma samples. These levels obviously give a more accurate demonstration of the drastic decrease of HDL in biliary obstruction than the levels reported in paper III.

Electroimmuno assay was also a very convenient and sensitive technique for measurement of the apoprotein distribution after ultracentrifugation or chromatography of lipoproteins. Despite the high salt concentration of fractions after zonal ultracentrifugation it was possible to apply the samples undiluted without any adverse effects observed, provided that low voltage (1-2 V/cm) was used at least during the first 15 minutes of the run. It should again be stressed that the results should not be considered as highly accurate measurements of apoprotein concentration for reasons already discussed.

Zonal ultracentrifugation

All runs were carried out in a Beckman model L2-65B ultracentrifuge. In most experiments we used a Ti-14 zonal rotor, which generates up to 172 000 g at 48 000 rpm and has a total volume of 665 ml, but occasionally a Z-60 rotor was used (I). The centrifugation technique was essentially performed as originally described by Wilcox et al. (107). Gradients, linear in density with respect to rotor volume, were made by mixing buffer (0.01 M Tris-HCl, pH 7.4, and 1 mM EDTA) and sodium bromide solution ($d = 1.30 \text{ kg/l}$) with two peristaltic pumps programmed by a curve follower.

A 600 ml NaBr gradient ($d = 1.00\text{-}1.29 \text{ kg/l}$) was employed for the separation of HDL. The density of the sample was adjusted to 1.295 kg/l with solid sodium bromide before introduction to the rotor. The centrifugation was carried out at 46 000 rpm (max RCF 158 000 g) at $+15^{\circ}\text{C}$ for 20 hours. The rotor was centre-unloaded at 3 000 rpm using a sodium bromide solution ($d = 1.30 \text{ kg/l}$) and UV-absorbance at 280 nm was continuously monitored. Fractions of 7.5 ml were collected and the densities of the fractions were checked by weighing 5 ml aliquots. The experimental conditions are fully described in paper I and additional pertinent data are given in papers III, V, and VI.

Other methods

Gel filtration. Plasma samples were chromatographed on a 5×90 cm column packed with Sephadex G-200 with particle size $20 \pm 2 \mu\text{m}$

obtained by dry elutriation of Sephadex G-200 super fine (108). Ascending chromatography in 0.05 M Tris-HCl, pH 7.4, containing 0.01 M EDTA and 0.2 M NaCl, was performed at +4°C and with a flow rate of 12 ml/h. The effluent was collected in 9 ml fractions and the absorbance at 280 nm of each fraction was measured.

Polyacrylamide gel electrophoresis. SDS-gel electrophoresis was performed in a discontinuous system as described by King and Laemmli (109).

The samples were reduced with 2-mercaptoethanol and heated at 90°C for three minutes prior to electrophoresis. Unreduced samples were used in some experiments in order to facilitate the estimation of apo A-II, which then appeared as dimer. Nine well-characterized proteins were used as molecular weight markers. Electrophoresis of the apoproteins was also carried out according to Davis (110) in 7.5% polyacrylamide gels containing 8 M urea after delipidation with 1,1,3,3-tetramethylurea (111). The latter method was used mainly as a complement to SDS-PAGE, since this method seems to give an underestimation of the content of apoproteins of a molecular weight below 10 000. After electrophoresis the proteins were stained with Coomassie Brilliant Blue R 250 for 3 hours. The gels were destained as described by Weber et al. (112) in the presence of an ion-exchange resin, Amberlite MB-1, in order to facilitate destaining.

To determine the relative amounts of apo A-I and apo A-II in HDL subclasses we used fluorescence-labelling of apoproteins combined with SDS-PAGE as described by Friedberg and Reynolds (113).

Lipid and protein analyses. The lipids were extracted from the lipoprotein fractions with chloroform:methanol 2:1 (v/v). The main lipid classes were separated by thin layer chromatography on silica gel using petroleum ether:ethyl ether:glacial acetic acid (90:10:1) as mobile phase. The lipids were localized by iodine vapour and eluted from the scraped areas with chloroform:methanol 1:1 (v/v). Triglycerides were determined by an enzymatic method with reagents from Boehringer Mannheim. Cholesterol and cholesteryl esters were quantitated by the method of Abell (114) or determined enzymatically (115). Phospholipids were determined by analysis of phosphorus in the lipid extract according to the method described by Bartlett (116). The fatty acid composition of cholesteryl esters was determined by gas liquid chromatography as described by Åkesson et al. (117). Protein concentrations were determined by the method of Lowry et al. (118) or a modification of this method (119).

in order to avoid falsely high results in turbid fractions.

Determination of cholesterol esterifying activity (LCAT activity).

Determination of lecithin:cholesterol acyltransfer was carried out as described by Stokke and Norum (120) (IV). Determination of the LCAT activity of zonal ultracentrifugation fractions was performed essentially as described by Nichols and Gong (121).

Electron microscopy. Prior to electron microscopy the lipoprotein fractions were dialyzed overnight against 0.1 M ammonium acetate. Aliquots of the dialyzed lipoproteins were mixed with an equal volume of 2% (w/v) sodium phosphotungstate, pH 7.4, and a droplet of this mixture was placed on a carbon-coated grid. The negatively stained lipoproteins were immediately examined under a Philips EM 300 electron microscope (IV, VI).

RESULTS AND DISCUSSION

Normal human HDL studied by zonal ultracentrifugation

The most widely used technique for separation of plasma lipoproteins is differential ultracentrifugation. This is a tedious and time-consuming procedure employing multiple centrifugations with adjustment of the density between the centrifugations. Complete separation of the various lipoprotein classes requires more than five days of centrifugation. The development of zonal rotors made it possible to overcome many of these drawbacks. Some earlier described zonal ultracentrifugation procedures have used a two-step procedure with changing of the gradient during the run (122, 123). Although no extensive studies of gradient shapes and centrifugation have been performed, the present work shows that it is possible to obtain a good separation of HDL in one single run (I).

Centrifugation for only 20 hours with a gradient linear to rotor volume (I, III, IV, V) gave a fairly good resolution of HDL into HDL₂ and HDL₃ well separated from VLDL-LDL and the bulk of plasma proteins (cf. paper I). The HDL₂ and HDL₃ were recovered at average densities 1.115 kg/l (1.105-1.120 kg/l) and 1.155 kg/l (1.140-1.165 kg/l), respectively, in ten centrifugations of human plasma. These densities indicate that the isopycnic state was not reached during runs, since the average density for HDL₂ is about 1.10 kg/l and for HDL₃ about 1.14 kg/l (11). The present results are essentially similar to those presented by Kostner et al. (122) and Patsch et al. (123), using zonal ultracentrifugation, although these authors reported a somewhat higher hydrated density for

HDL₂ and HDL₃. This could simply be explained by differences in running conditions, i. e. shape of the gradient and gravitational force.

The fractions from the runs were tested with electroimmuno assay against the following antisera: anti-apo A-I, anti-apo A-II, anti-apo B, and anti-albumin. No contamination of HDL fractions with LDL (LpB) or albumin could be demonstrated (cf. Fig. II, paper I). The distribution of apo A-I followed the 280 nm curve in the HDL₂ and HDL₃ regions.

Small amounts of apo A-I were also found between HDL₃ and the plasma proteins (VHDL) and trace amounts were even recovered associated with the plasma protein fraction after zonal ultracentrifugation. The amounts of apo A-I in the latter fraction appeared to be less than 5% of the apo A-I in the original plasma, as judged from the results of electro-immuno assay. Similar results were obtained by Kostner (122). On the other hand considerably higher contents of apo A-I were present in the fraction $d > 1.21$ kg/l after differential ultracentrifugation of human plasma (124, 125).

Immunoreaction with anti-apo A-II was found mainly in HDL₃; very faint precipitates were also seen in fractions from the VHDL region but not in the fractions containing plasma protein. The distribution of apo A-II in the HDL₂ was difficult to measure, since the immunoprecipitates became gradually fuzzy and eventually vanished in the lighter part of this fraction. This phenomenon may be explained by a decreasing content of apo A-II or by inaccessibility of apo A-II determinants in the HDL₂ region. The first explanation is the most likely one since a low content of apo A-II in HDL₂ was also found with polyacrylamide gel electrophoresis (III). The present observations of the distribution of apo A-I and A-II in the zonal ultracentrifugation fractions are similar to those reported earlier by Kostner et al. (122).

Recentrifugation of the HDL₂ and HDL₃ fractions, under the same conditions as in the first separation, showed that the distribution of these subclasses remained essentially unchanged as judged from the 280 nm absorbance curve and from the distribution of apo A-I (I). Only trace amounts of material reactive with anti-apo A-I were present in other density regions (less than 5% of the apo A-I content of the lipoproteins subjected to recentrifugation). This loss is of the same order as that reported by Patsch et al. (123) but apparently smaller than that found by Scanu and Granda (126) using differential ultracentrifugation. It is noteworthy that little dissociation seems to occur in zonal ultracentrifugation, despite the exposure of HDL to twice as high concentration of the chaotropic bromide

ion (127) in this technique compared with differential ultracentrifugation.

The lipid and protein composition of HDL₂ and HDL₃ separated by zonal ultracentrifugation was consistent with earlier results obtained by differential centrifugation with one exception. The proportion of apo A-I to apo A-II was higher in HDL₂ obtained by zonal ultracentrifugation as judged from visual inspection of the electrophoretic patterns in urea- or SDS-polyacrylamide gels (III). This was further confirmed with the technique described by Friedberg and Reynolds (113) using fluorescent labelled apo-proteins in SDS-PAGE. The molar ratio of apo A-I to apo A-II in normal HDL₂ was about 10:1 (Table IV). It is obvious from these results that normal HDL₂ obtained by zonal ultracentrifugation has a low apo A-II content and this explains the vanishing precipitates on electroimmuno assay of the fractions in the lighter parts of the HDL₂ density region.

The proportions of apo A-I to apo A-II in HDL₂ and HDL₃ is a matter of controversy as illustrated by Table IV, summarizing recent investigations including data from the present work. The diverging values may be explained by slight differences in the ultracentrifugation procedures as well as in the techniques used for apoprotein assays. It is evident, however, that the zonal ultracentrifugation procedures gave similar ratios independent of assay technique. The reasons for the differences in apoprotein composition of HDL₂ obtained by zonal ultracentrifugation and differential centrifugation are not known and further studies are needed to clarify this discrepancy. It should be noted, however, that zonal ultracentrifugation in a non-ionic gradient medium (metrizamide), although separating HDL₂ and HDL₃ less efficiently, seems to give HDL₂ with a similar low content of apo A-II in HDL₂, which makes salt effects less likely as an explanation (unpublished experiments).

In contrast to the clear separation of the main lipoprotein classes (VLDL, LDL, HDL) the zonal ultracentrifugal pattern revealed no distinct peak in the VHDL region (i.e. the lipoproteins recovered in the density interval of 1.21-1.25 kg/l by differential ultracentrifugation). This region was instead represented by an extended plateau between HDL₃ and the plasma proteins, seen after prolonged centrifugation of plasma for 48-56 hours (I). No systematic studies of the lipoproteins in the VHDL fraction have been performed in the present study, but the protein and lipid composition was found to resemble that of HDL₃. The protein content was about 75%, increasing from 67% at 1.20 kg/l to 85% at 1.24 kg/l. Fractions with higher density were heavily contaminated with the bulk of plasma proteins. The protein part of VHDL consisted almost entirely of apo A-I. The VHDL

TABLE IV

Comparison of the molar ratio of apo A-I to apo A-II in normal human HDL₂ and HDL₃ isolated with different methods

	Curry et al. (128)	Cheung et al. (106)	Friedberg and Reynolds (113)	Kostner et al. (122)	Danielsson et al. (129)
Isolation procedure of HDL ₂ and HDL ₃	Sequential ultracentrifugation	Sequential ultracentrifugation	Sequential ultracentrifugation	Zonal ultracentrifugation	Zonal ultracentrifugation
Analytical methods	Immuno-chemically EIA	Immuno-chemically Radial immuno-diffusion	Fluorescence labelled apo-protein SDS-PAGE	Immuno-chemically Radial immuno-diffusion	Fluorescence labelled apo-proteins SDS-PAGE
Molar ratio of apo A-I to apo A-II in HDL ₂	1:1	3.5:1	2:1	10:1	10:1
in HDL ₃	1:1	2:1	2:1	2:1	2:1

were also found to contain the plasma LCAT activity (I) in agreement with earlier studies using differential ultracentrifugation (130).

The separation of human HDL into subclasses may be improved, by the use of higher gravitational force during zonal ultracentrifugation. By this means Laggner et al. (131) recently resolved HDL into three distinct peaks, differing in lipid and protein composition. A partial separation into three HDL subfractions has also been obtained by gradient centrifugation in a swing-out rotor (132). Analyses of the lipoprotein material in the latter fractions with gradient polyacrylamide gel electrophoresis gave evidence for a particle size heterogeneity of each of the fractions. These results indicate a pronounced heterogeneity of HDL which is only partly reflected by the zonal ultracentrifugal procedure used in the present study (cf. paper IV).

The density distribution of HDL subfractions differ considerably for various mammalian species. In earlier studies with zonal ultracentrifugation it was shown that canine HDL (133), like human HDL, can be subfractionated into HDL₂ and HDL₃, while no such subfractionation was obtained for porcine plasma (134). Paper VI presents another HDL pattern occurring in rat's plasma, characterized by the presence of a main HDL peak and an additional peak with an apparent density of 1.08 kg/l (HDL₁). These lipoprotein fractions had similar lipid composition, while the apoprotein composition of the HDL₁ fraction was characterized by a predominance of apo E over apo A-I. The HDL₁ fraction described in paper VI is similar to the rat HDL₁ quite recently described by Weisgraber et al. (135) using differential ultracentrifugation in combination with Geon-Pevicon block electrophoresis. The biological significance of these differences in HDL distribution between mammalian species is not known, but it is obvious that a better knowledge of the species differences discussed is important in studies of HDL metabolism.

HDL in chronic alcoholics

The lipoprotein changes in 38 males, classified as chronic alcoholics, were studied after recent debauches (V). No consistent changes in plasma triglycerides or cholesterol were found, which rules out more profound alterations of VLDL and LDL (cf. Table I in paper V).

On the other hand, two thirds of the subjects had increased HDL cholesterol concentrations compared to a control group of apparently healthy men in a corresponding age group. HDL (measured immunochemically) were elevated in about the same percentage of the patients (but not

in the identical individuals, see below).

The parallel determination of HDL cholesterol and immunochemical quantitation of HDL made it possible to obtain an expression of the cholesterol-protein ratio of HDL. This was fairly constant in the controls but showed a considerable variation in the alcoholics (cf. Fig. 1 in paper V). It is evident from these data that determination of only one of the components, e.g. cholesterol or apoprotein(s), might give limited or possibly misleading information on changes of HDL concentrations. For example, an increase of cholesterol-rich particles (e.g. HDL₂) will be more readily detected by measurement of HDL cholesterol than by apoprotein determination, while comparable alterations in HDL₃ concentration are less impressive if expressed in terms of HDL cholesterol. More complete information on changes in HDL levels, especially under pathophysiological conditions, is thus obtained by parallel measurements of HDL cholesterol and HDL apoprotein.

The apparent variation in the composition of HDL was reflected by the zonal ultracentrifugation patterns of plasma from alcoholics. The distribution of HDL analyzed in eight alcoholics was very divergent. The high HDL levels were manifested as increased concentrations of HDL₂, HDL₃ or both of these as judged from the light absorption at 280 nm. Preliminary data indicated that there were no gross abnormalities in lipid or protein composition of the HDL subclasses. After cessation of alcohol intake the changes in HDL were essentially normalized within one week as shown by zonal ultracentrifugation and determinations of HDL cholesterol or HDL protein (V), in accordance with the results of an earlier study (90).

In a current study on HDL changes after alcohol debauches, the zonal ultracentrifuge patterns of five additional alcoholics have been studied. In this study the quantitative relationship between HDL₂ and HDL₃ was estimated by measurement of the protein content of these subclasses from the absorption at 280 nm, assuming normal lipid-protein ratios in HDL₂ and HDL₃. The values were calculated from estimated values of $A_{1\text{ g/l}}^{280\text{ nm}}$ for the protein part of HDL₂ and HDL₃ found to be 1.67 and 1.46, respectively. The results are shown in Table V and the corresponding zonal ultracentrifuge patterns are shown in Fig. 1. These results confirm those presented in paper V with regard to the various HDL patterns found. As seen in Table V three of the five patients showed an increased ratio HDL₂:HDL₃, evidently due to an increase of the HDL₂ levels. The abnormal distribution of HDL subclasses was normalized in one week. Patient (VM) had a probable increase of HDL₃, which remained virtually unchanged, whereas

TABLE V

Plasma levels and proportions of HDL₂ and HDL₃ in five male alcoholics admitted to an alcohol clinic. The calculated values are based on the density intervals 1.090-1.125 kg/l for HDL₂ and 1.125-1.185 kg/l for HDL₃. The protein contents of HDL₂ and HDL₃ were assumed to be 41% and 55%, respectively.

	Day after admission	HDL ₂	HDL ₃	HDL ₂ : HDL ₃
Patients				
VM	1	0.63	3.09	1 : 4.9
	9	0.76	2.93	1 : 3.9
SE	1	1.06	1.70	1 : 1.6
	7	0.23	2.31	1 : 10.1
KE	1	0.35	2.33	1 : 6.7
	7	0.38	2.13	1 : 5.6
SC	1	1.36	1.3	1 : 1.0
	8	0.17	1.8	1 : 10.6
LN	1	0.90	2.0	1 : 2.2
	9	0.29	1.8	1 : 6.2
Normal male		0.44	1.70	1 : 3.9
Reference ^x		0.53±0.48	2.22±0.31	1 : 4.2

^x Reference values obtained by analytical ultracentrifugation given for comparison (14).

patient (KE) had no gross abnormalities in the HDL distribution. Further studies on the quantitative determination of HDL subclasses including zonal ultracentrifugations of plasma from a representative reference group are needed for a final evaluation of the subclass changes.

The cause of the HDL alterations in alcoholics is not understood. It is reasonable to assume that the intake of alcohol per se is responsible for the effects on HDL, since a rapid normalization takes place after alcohol withdrawal (90, V). The time for normalization is roughly consistent with the known half-life of plasma HDL of 4-5 days (15). Although signs of impaired liver function were present in the majority of patients studied there was no correlation between liver tests and the increase of HDL. Estrogen

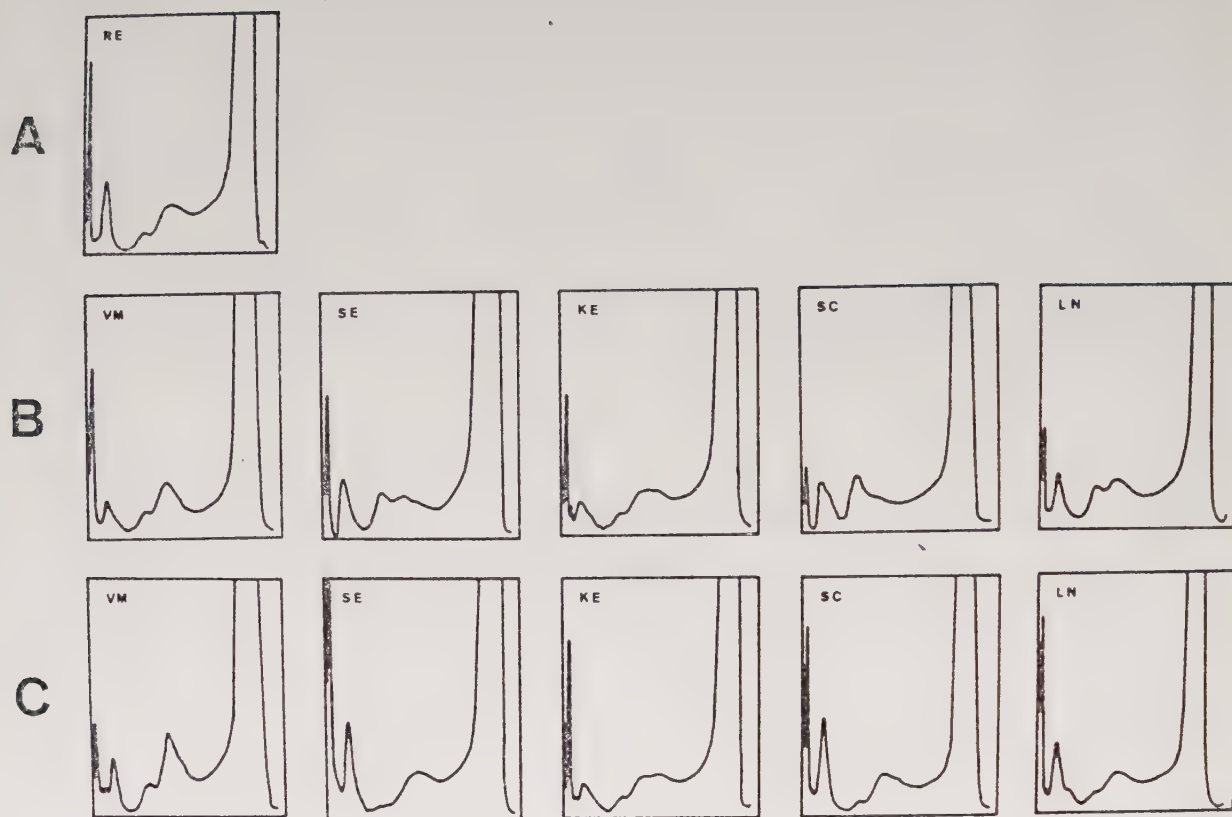


Fig. 1. Lipoprotein patterns obtained by zonal ultracentrifugation of plasma from five male chronic alcoholics. The corresponding levels of HDL subclasses are shown in Table V. Zonal ultracentrifugation was performed as described in paper I for separation of the main density classes. Plasma from a healthy male run for comparison (A), patterns obtained on day 1 (B) and one week later (C). Equal amounts of plasma (18 ml) were centrifuged in all cases except in B (VM) where only 13 ml was used.

influence, which is known to cause an increase in HDL (34, 35) and particularly HDL₂, was also taken into account since increased circulating estrogens have been considered responsible for the decreased testicular function, gynecomastia and palmar erythema often found among alcoholics (136). An estrogen influence causing increase in HDL should, however, also be associated with considerable increases of plasma ceruloplasmin and transferrin (137). The finding of normal ceruloplasmin and total iron-binding capacity thus rules out this possibility.

The HDL increase seems to be the result of habitual intake of alcohol for some time. Occasional consumption (80 g) does not cause any demonstrable increase in HDL (unpublished observation). On the other hand, as

demonstrated by Belfrage et al. (88), a slight but significant increase in HDL was obtained in healthy volunteers after a daily intake of approximately 63 g of alcohol as beer for five weeks.

The mechanism(s) behind the HDL alterations after alcohol intake remain so far speculative. Possible mechanisms operating might be an increased formation of plasma HDL or a diminished removal of circulating HDL, but the known relation between HDL and VLDL (triglyceride) metabolism must also be taken into account. Although no statistically significant inverse correlation was found between HDL and triglyceride levels (V), current studies of the enzyme activities connected with the metabolism of lipoproteins in plasma have shown increased levels of lipoprotein lipase activity in postheparin plasma of chronic alcoholics, while hepatic lipase activities were normal (unpublished results). No changes in LCAT activities were found. The increase in lipoprotein lipase activity in postheparin plasma is an observation which is consistent with the earlier demonstration that long-term moderate ethanol intake in normal individuals leads to a significant increase in adipose tissue lipoprotein lipase activity (91) and a concomitant decrease of plasma triglycerides. The observations of increased lipoprotein lipase activity suggests that the increase in plasma HDL concentration in alcoholics is related to an accelerated turnover of triglyceride-rich lipoproteins. This interpretation is consistent with the observation that heparin-induced lipolysis in vivo as well as incubation of VLDL with postheparin plasma in vitro is associated with an increase in HDL (α -lipoprotein) concentrations (39). The explanation of the marked changes in density distribution mirrored by the altered proportions of the subclasses HDL₂ and HDL₃ must await further studies of the metabolic significance of these subclasses.

The use of laboratory tests has become important in the detection of overconsumption of alcohol or covert alcoholism. Determination of plasma γ -glutamyltransferase activity, which is often increased in alcoholics (138, 139), has been suggested for this purpose. However, since plasma γ -glutamyltransferase activity is normal in about 25-50% of alcoholics (90, 139, V) and increased in many conditions besides alcoholism, there is need for additional laboratory procedures in the detection of alcohol abuse. As shown in paper V elevated levels of HDL cholesterol or HDL protein were found in 84% of the alcoholics. A combination of plasma γ -glutamyltransferase determinations and plasma HDL measurements (determination of HDL cholesterol and protein), on the other hand, revealed abnormal values of at least one of these variables in 95% of the alcoholics (36 of 38

patients). The assessment of the usefulness of this test combination for the detection of alcoholism will require further studies of its diagnostic sensitivity and specificity in other populations than known male chronic alcoholics.

HDL in human cholestasis

Alterations may affect all lipoprotein classes in conditions with biliary obstruction. The most well-known change in human cholestasis is the appearance of an abnormal LDL component, LpX (140). It is also known that α -lipoprotein (HDL) may be markedly decreased, especially in cases with long-standing biliary obstruction, associated with a pronounced reduction of LCAT activity (74, 141). The α -lipoprotein reduction has been studied in the present work and will be discussed below.

Electrophoretic and immunochemical studies showed marked alterations of α -lipoprotein in plasma from patients with cholestasis (II). In lipoprotein electrophoresis, the α -lipoprotein and pre- β -lipoprotein bands were drastically reduced or even absent, and there was a broad band in the β -region (II). Despite the faint staining of α -zones in electrophoresis, EIA against anti- α -lipoprotein demonstrated only a moderate decrease of α -lipoprotein, but it was noted that the immunoprecipitates differed from those obtained with normal plasma, showing less distinct "rockets". Furthermore, the precipitates were not stainable with Sudan Black. Similar findings have been reported by others, using EIA of plasma α -lipoproteins from patients with acute hepatitis (142):

The changes of α -lipoprotein indicated by the abnormal immunoprecipitates were confirmed by crossed immunoelectrophoresis (II, III). A complicated precipitation pattern compared to that of normal α -lipoprotein was obtained with 3-4 peaks between the α - and pre- β -region. This pattern was very similar to that found in familial LCAT deficiency (II, unpublished observations). The distribution of the most anodal peak corresponded to that of normal α -lipoprotein, while the position of the other 2-3 peaks, not demonstrable in normal plasma, suggested compositional abnormalities in these HDL particles. The latter components were further studied using crossed immunoelectrophoresis with an intermediate gel demonstrating that one of the components, which was not consistently present in all patients, had an immunoreactivity against anti-apo A-II, while apo A-I was apparently missing (III). The presence of such lipoprotein particles in cholestasis has been suggested in earlier studies by Seidel et al. (77), who found a dissociation of the main HDL apoproteins from cholestatic HDL in immunoelectrophoresis. No detailed information on the composition and

properties of these presumed apo A-II-containing lipoprotein particles has so far been presented. Recently, HDL particles containing only apo A-II have been isolated and characterized from patients with familial HDL deficiency (Tangier's disease) (143).

The most cathodal component in cholestatic plasma reacting with anti-HDL also gave a distinct immunoprecipitate with anti-apo C-I. Further studies of this precipitate in crossed immunoelectrophoresis (III) indicated that it was formed by an abnormal cholestatic lipoprotein (HDL₁-C). This lipoprotein was previously shown to contain considerable amounts of apo C-I (144).

The HDL alterations were further studied by zonal ultracentrifugation (III, IV). A marked decrease of material in the normal HDL₂-HDL₃ regions was found and the normal delineation of these subclasses disappeared. According to the immunochemically measured distribution of apo A-I, a small peak was found which had a higher density (1.18 kg/l) than that of normal HDL₃ (III, IV). In addition a small but distinct fraction with an apparent density of 1.08 kg/l, tentatively called cholestatic HDL₁ (HDL₁-C), was found. This fraction was well separated from LDL.

The time-course for the appearance of these changes was studied in a few patients (III). The first change was the disappearance of HDL₂ followed by a decrease of HDL₃. The appearance of the HDL₁-C fraction was a later event which should be demonstrated in patients with extrahepatic obstructive jaundice about two weeks after its probable onset. After surgical relief of the biliary obstruction, the HDL₁-C fraction disappeared within one week followed by normalization of HDL₃ and finally HDL₂ within a period of three weeks. This order of events might possibly reflect a sequential transformation of HDL₁ → HDL₃ → HDL₂.

Gel filtration of cholestatic plasma revealed HDL patterns consistent with those obtained by ultracentrifugation. There was a markedly abnormal HDL distribution, which was determined immunochemically (EIA) using anti-HDL (III). Generally two completely resolved HDL peaks were obtained. One small peak eluted slightly after the void volume, while the other main peak eluted later than the main part of normal HDL (cf. its high density in zonal ultracentrifugation). The material from the first and second peaks behaved in the same way on crossed immunoelectrophoresis as HDL₁-C and normal HDL, respectively. These results suggest that HDL₁-C is not an artefact generated by changes of the HDL particles during the zonal ultracentrifugation step. A similar separation of isolated cholestatic HDL in a large and a low particle size fraction was obtained by Blomhoff, also

using Sephadex G-200 (145).

Electron micrographs of HDL₁-C isolated by zonal ultracentrifugation clearly illustrated ultrastructural abnormalities; the only particles seen were bilamellar discs stacked in long chains. The discs measured 15 to 20 nm in diameter and 3 to 5 nm in thickness (IV). No particles were found that resembled normal HDL, which consist of spherical particles with diameters ranging from 4 to 14 nm (cf. Table I). The dimensions of the HDL₁ particles, which explain the low distribution volume on gel filtration with Sephadex G-200, are similar to the disc-shaped HDL particles found by others in cholestatic plasma (79, 146) or plasma from patients with familial LCAT deficiency (79, 80).

The lipid composition of cholestatic HDL subfractions was different from that found in normal HDL (III). A high content of polar lipids (phospholipids and unesterified cholesterol) was found especially in HDL₁-C, as might be expected from its morphology.

The phospholipid composition of HDL₁-C showed no important differences to that of normal HDL (IV). Phosphatidylcholine was the main component, and the lysolecithin content was not markedly changed. On the other hand, the fatty acid pattern of the minor cholesteryl ester fraction was remarkable. Palmitic acid was the predominant component, whereas only small amounts of linoleic acid were found (IV). This pattern indicates that the formation of the cholesteryl esters was mainly achieved by the action of enzymes other than LCAT, which preferably catalyzes the transfer of linoleic acid from phosphatidylcholine to cholesterol (47). Similar fatty acid patterns were found in whole plasma from patients with familial LCAT deficiency (147).

The apoprotein composition of cholestatic HDL₁-C and HDL₂ fractions also differed considerably from that of normal HDL, whereas cholestatic HDL₃ was more like its normal counterpart. The HDL₁-C fraction was characterized by a high content of apo E, while other apoproteins (i.e. apo A and apo C) were present in smaller amounts (III, IV). The ratio apo E:apo A-I around 4.5:1 calculated from the results of apoprotein determinations after fluorescent labelling (IV) might be an underestimation with regard to the comparatively low content of ε-amino groups available for binding of fluorescamine in apo E (17-19). In a few patients with pronounced cholestasis of long duration apoproteins other than apo E were only present in trace amounts. No immunochemically detectable apo B was found in HDL₁-C, a finding that excludes contamination of LDL (LpB). Similar findings of high concentrations of apo E have been reported in HDL₂ of

cholestatic plasma (146), in HDL from patients with familial LCAT deficiency (42, 146), and quite recently in HDL from patients with alcohol hepatitis (148).

There was generally a higher content of apo A-II than of apo A-I in HDL₁-C, as judged from densitometric scanning of urea polyacrylamide gels (144) or fluorescence measurements of fractions after SDS-PAGE of fluorescent labelled apoproteins (unpublished results). There was more apo C in HDL₁-C than in normal HDL, and in some preparations there was a marked, selective increase of apo C-I and apo C-III₂ (144).

The observation that apo A-I and apo E in the HDL₁-C fraction obtained by zonal ultracentrifugation did not have identical distributions (cf. Fig. 1 in paper IV) suggested heterogeneity of the discoidal particles in this fraction with regard to their apoprotein composition. To study this possibility in greater detail immunochemical methods were used, such as immunosorbent chromatography and crossed immunoelectrophoresis with an intermediate gel (IV).

When HDL₁-C was chromatographed on immobilized anti-A-I about two thirds of the apo E was found to pass the column unretained. It was shown that the unbound lipoprotein was devoid of any measurable apo A-I indicating the presence of lipoprotein particles containing apo E but not apo A-I. The unbound fraction contained, besides apo E, small amounts of apo A-II and apo C.

The results mentioned above might indicate the presence in HDL₁-C of lipoprotein particles containing only one apoprotein constituent. With a modification of crossed immunoelectrophoresis involving the use of intermediate antibody-containing gels (99) it was possible to demonstrate that HDL₁-C contained lipoprotein particles with apo E as the only apoprotein, since they passed unimpeded through gel layers containing antibodies to apo A-I, apo A-II and apo C (IV). It was found that about half of the apo E-containing particles passed through such intermediate gels, indicating that a considerable part of the particles contained virtually only apo E (IV). This interpretation of the results obtained with the intermediate gel technique is only relevant under the assumption that no apoproteins are dissociated from HDL₁-C particles under electrophoresis, and that all three C proteins are present in the same lipoprotein particles as suggested by Kostner and Alaupovic (149), since only anti-apo C-I was used in the present work.

The cause of the apparent LCAT deficiency in biliary obstruction is not known. Although normal LCAT activities may be found in cholestasis

(150) it seems quite evident that at least long-standing obstructive jaundice is associated with decreased plasma cholesterol esterification (74, 141) and concomitant changes of HDL as described in papers III and IV. The low LCAT activity is presumably due to low production (or secretion) of the enzyme per se and not to the presence of inhibitory substances like bile salts (74), or to deficiency of apo A-I, serving as cofactor in the LCAT reaction (23). Although low amounts of apo A-I were found in cholestatic plasma, similar levels measured in plasma from patients with Tangier disease were not associated with LCAT deficiency, indicating that such small amounts of apo A-I are sufficient to maintain the conversion of cholesterol to cholesteryl esters (143).

The low concentration of LCAT might be explained by progressive liver damage induced by the biliary obstruction. Several studies have documented low plasma LCAT activity and/or marked alterations of HDL in patients with acute hepatitis of various etiology (76, 151, 152, 153). On the other hand, anicteric patients with liver cirrhosis may have moderately to severely decreased levels of HDL, and a moderate reduction of LCAT activity, without the qualitative changes in HDL as judged from results of crossed immunoelectrophoresis (unpublished results). The zonal ultracentrifugation patterns of HDL in three cirrhotic patients with low levels of HDL (measured immunochemically as the apo A-I concentration) are shown in Fig. 2, demonstrating HDL distributions which are quite different from those in biliary obstruction. The low HDL levels in these patients might possibly be explained by a shift from intra- to extravascular space, preferably affecting the smaller HDL₃ particles.

The probable occurrence of a lipoprotein E particle in cholestatic plasma is interesting in the light of the recent concept of Osborne and Brewer postulating the existence of "primary" lipoprotein particles, containing a single apoprotein or apoprotein family (13). Further studies with the use of several immunosorbents including immobilized anti-apo A-II and total anti-apo C would be helpful to ensure the isolation of LpE. No detailed studies of the LpE fraction obtained by immunosorbent chromatography of HDL₁-C on anti-apo A-I columns have so far been performed. Electron microscopic studies have revealed, however, that it consists of disc-shaped particles of about the same size as those in the unfractionated HDL₁-C.

The present results confirm and extend earlier studies reporting on the presence of apo E in HDL from patients with low LCAT activity of primary (42, 146) or secondary (146) forms. It is not known why apo E occurs

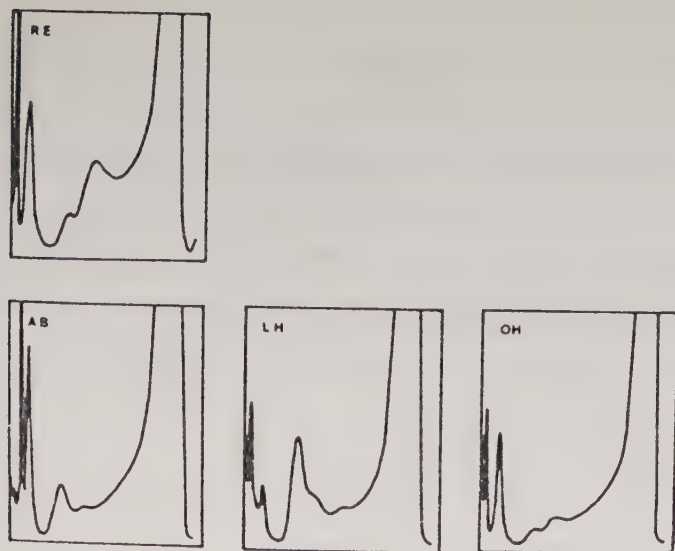


Fig. 2. Zonal ultracentrifugation patterns of 35 ml plasma from a healthy male (RE) and from two females (AB; LH) and one male (OH) with established liver cirrhosis. All three patients had decreased levels of HDL measured immunochemically with anti-apo A-I (AB: 50%, LH: 54%, and OH: 59% of the mean level for healthy men). Two of the patients had reduced LCAT activities; the percentage of cholesterol esterified per hour was 2.1 (AB), 3.9 (LH), and 1.8 (OH) (normal range 3.5-5.0).

in HDL under these conditions, but the phenomenon is evidently related to the low LCAT activity, since it has been reported that addition of purified LCAT to plasma from patients with LCAT deficiency decreased the content of apo E in HDL with a concomitant increase of apo E in VLDL (42). The possibility of isolating LpE or at least lipoproteins with almost only apo E as protein constituent from easily available material such as cholestatic plasma facilitates further studies on the normal transformation of this apparently "primary" lipoprotein particle.

The present results should also be put in relation to some interesting findings in experimental animals. Hamilton et al. (40) isolated "nascent" HDL from rat liver perfusates in the presence of an inhibitor of LCAT activity. These nascent HDL particles are very similar to human HDL₁-C; both the rat lipoprotein and HDL₁-C are disc-shaped, contain mainly polar lipids (and thus only low amounts of cholesteryl esters) and have apo E as their major protein constituent. It is quite possible that the cholestatic HDL₁ is the human analogue of rat nascent HDL.

In another model using rats Sabesin et al. (154) showed that a single intraperitoneal injection of D-galactosamine, inducing a temporary liver

damage, produced striking compositional and morphological changes in all lipoprotein classes in association with a rapid and reversible LCAT deficiency. The observed alterations, including a reduction of HDL and the formation of disc-shaped HDL particles, were remarkably similar to changes found in states of LCAT deficiency. However, little information on changes in the apoprotein composition of HDL was given in their communication. In order to study possible changes in apoprotein composition during induced liver-biliary damage in rats a similar study was undertaken. Preliminary results showed that galactosamine treatment resulted in a marked decrease of LCAT activity and a virtual disappearance of α -lipoprotein in lipoprotein electrophoresis. Studies of plasma from treated rats with zonal ultracentrifugation also showed a marked decrease of HDL in comparison with plasma from rats used as controls. However, the interpretation of the patterns was complicated by the fact that an additional lipoprotein fraction (HDL₁) was found between LDL and HDL in the control animals (VI). This fraction was isolated and was found to contain a major protein component which is most probably identical with apo E as judged from its apparent molecular weight on SDS-polyacrylamide gel electrophoresis. In accordance with the results of others (155) apo E was also present in small amounts in the main HDL fraction. Despite the similarities in apparent density and apoprotein composition, the HDL₁ lipoprotein was different from the "nascent" rat HDL (and human HDL₁-C), since it had a lipid composition similar to normal HDL and appeared as spherical particles by electron microscopy (VI). The biological significance of this lipoprotein is not known, but further studies of its fate might be of great value in the elucidation of the metabolism of LpE.

SUMMARY

High density lipoproteins have been studied by zonal ultracentrifugation and immunochemical methods in normal subjects in comparison with two patient groups with changed plasma HDL levels: chronic male alcoholics and patients with long-standing biliary obstruction.

Zonal ultracentrifugation of plasma was found to give a separation of the HDL subclasses HDL₂ and HDL₃ after 20 hours' centrifugation with minute losses of apo A-I. After prolonged centrifugation for 48 hours, a VHDL fraction containing the LCAT activity in plasma could be separated. No differences in lipid and apoprotein composition could be noted for the HDL subclasses obtained by zonal ultracentrifugation compared with those isolated by differential ultracentrifugation, with the exception of a higher molar apo A-I:apo A-II ratio in HDL₂ isolated by zonal centrifugation.

HDL were moderately increased in two thirds of male chronic alcoholics admitted to an alcohol clinic after a recent drinking bout. After cessation of alcohol intake normalization of the HDL levels occurred in one week. Zonal ultracentrifugation of plasma from the alcoholics did not reveal any uniform HDL subclass patterns. Half of the patients had an essentially selective increase of HDL₂, while the others had increased HDL₂ and/or HDL₃ levels. The subclass distribution was reflected by the results of determinations of HDL protein (apo A-I) and HDL cholesterol; the patients with increased HDL₂ had a more pronounced increase of HDL cholesterol compared with apo A-I.

It was demonstrated that HDL determined as HDL protein and/or HDL cholesterol may be used as a complement to serum γ -glutamyltransferase determinations in the detection of heavy alcohol consumption. Further studies are needed to confirm the value of HDL determinations for this purpose.

The marked reduction of HDL levels in patients with long-standing biliary obstruction was confirmed by plasma lipoprotein electrophoresis showing an apparent absence of α -lipoprotein in most cases. Crossed immunoelectrophoresis of cholestatic plasma against anti-HDL revealed marked alterations in the HDL distribution similar to those found in familial LCAT deficiency.

The HDL changes in cholestasis were further studied by zonal ultracentrifugation, demonstrating a marked reduction of normal HDL₂ and HDL₃. In addition, an HDL₁ fraction, not present in normal plasma, was found. This lipoprotein, called cholestatic HDL₁ (HDL₁-C) differed

markedly from normal HDL components. It consisted of disc-shaped particles rich in polar lipids (phospholipids and unesterified cholesterol). The fatty acid composition of the minor cholesteryl ester fraction was characterized by a low content of linoleic acid, indicating that the cholesteryl esters had mainly been formed by enzymes other than LCAT.

The apoprotein composition was characterized by a high content of apo E, while only small amounts of apo A-I were found. The different distributions of apo E and apo A-I in the HDL₁-C region indicated a compositional heterogeneity of HDL₁-C. Immunochemical studies of HDL₁-C suggested the presence of particles containing only apo E in the protein part (LpE).

The HDL changes in biliary obstruction are very similar to or even identical with those described in familial LCAT deficiency, suggesting that the apparent LCAT deficiency in long-standing cholestasis is the main reason for the HDL alterations. Furthermore, the chemical composition of the HDL₁-C fraction is remarkably similar to that obtained for "nascent" HDL isolated from perfused rat liver in the presence of an LCAT inhibitor.

Comparative studies of HDL in rat plasma also revealed the presence of an HDL₁ fraction with apo E as its major apoprotein. However, the lipid composition was similar to that of the main rat HDL with the major part of the cholesterol esterified and furthermore the lipoprotein appeared as spherical particles in the electron microscope. Consequently the normal rat HDL₁ is not identical with "nascent" HDL, but the possible relationship to the latter particles remains to be established.

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SEPARATION OF PLASMA LIPOPROTEINS BY ZONAL ULTRACENTRIFUGATION

B. Danielsson[†], R. Ekman^{*} and B.G. Johansson^{*}

Departments of Biochemistry[†] and Clinical Chemistry^{*},
University of Lund, Lund, Sweden

ABSTRACT

Procedures for the separation of plasma lipoprotein classes and subclasses by zonal ultracentrifugation are described. The main density classes, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL), in plasma can be separated in a single run for 20 hours. For the isolation of VLDL-LDL a centrifugation time of only 90 minutes is needed. Separations can be performed on plasma volumes varying from 10 to 400 ml in the Ti-14 rotor used; VLDL can in this way be isolated from 400 ml plasma in 30 minutes. The advantages and disadvantages of zonal ultracentrifugation in comparison with the commonly employed differential ultracentrifugation for separation of lipoproteins are discussed.

INTRODUCTION

The ultracentrifuge flotation technique has proved to be an indispensable tool for the preparation of plasma lipoproteins. The flotation is generally achieved by differential centrifugation at fixed densities, resulting in a separation of the well-known density classes VLDL, LDL, and HDL (1, 2). The differential centrifugation technique is, however, a tedious and time-consuming procedure, requiring repeated centrifugations. Furthermore the use of fixed densities may give incomplete or even misleading information on lipoprotein distribution, especially in studies on plasma lipoproteins in pathological conditions or in species other than man. These disadvantages are at least partly overcome by centrifugation in density gradients, but since only minute amounts of material can be isolated in swinging bucket rotors earlier available, this method has been little used.

The high performance, large scale zonal rotors (3) have offered new possibilities for the isolation of lipoprotein classes and subclasses in a simple way with a considerably increased resolution and capacity. The method has been successfully applied to the separation of human lipoproteins of various density classes (4-13) and has also been employed in studies of other mammalian lipoproteins (14-17).

The present communication gives a comprehensive view of the potentials of the zonal ultracentrifugation technique for the separation of lipoprotein classes and subclasses. The advantages and drawbacks of the method will be discussed.

MATERIALS AND METHODS

Human plasma as well as plasma from pigs and dogs were used in this work. Venous blood samples were drawn with Na_2EDTA (1 mg/ml) as anticoagulant after fasting for 12-14 hours. Plasma was separated within one hour and stored at $+4^\circ\text{C}$ for at most 6 hours before zonal ultracentrifugation.

The immunochemical distribution of apoproteins in zonal ultracentrifugation fractions was studied by electroimmuno assay (18) against antisera to various lipoproteins or apoproteins as described elsewhere (19).

Pooled fractions from zonal ultracentrifuge experiments were concentrated by ultrafiltration on Amicon filters (UM 2 or UM 10) or by dialysis against a concentrated solution of polyethylene glycol with a medium molecular weight of 20 000. The latter procedure is known to give a minimal loss of protein (9). The dialysis was performed in Spectrapor tubings with a cut-off of MW 3 500. The apoprotein composition of the fractions was studied by polyacrylamide gel electrophoresis in sodium dodecylsulphate (20) or 6 M urea (21).

Protein determinations were performed with a modified Lowry procedure (22).

Lecithin:cholesterol acyltransferase (LCAT) activity was determined by the method described by Nichols and Gong (23).

Zonal ultracentrifugation procedures

All chemicals used were analytical grade. Density gradient materials included sodium bromide and sucrose from Mallinckrodt, deuterium oxide from Merck (Uvasol) and Metrizamide ^(R) from Nyegaard & Co. A/S,

Oslo. All solutions used for gradient formation contained 10 mM Tris-HCl, pH 7.4, and 1 mM Na₂EDTA. The density gradients were prepared with a programmable gradient machine from one light solution and one dense solution (Fig. 1). The light solution was used also as overlay and the dense solution for the cushion, i.e. the part of the rotor content nearest the peripheral rotor wall. All solutions were degassed before use and the sodium bromide solutions were also filtered to remove possible traces of insoluble matter.

Equipment: All centrifugations were performed in a Beckman L2-65B centrifuge using Ti-14 or Z-60 rotors. The Ti-14 rotor has a volume of 665 ml, a maximum path length of 53 mm and $g_{\text{max}} = 172\,000$ at 48 000 rpm, while the Z-60 rotor with a path length of 48 mm contains 330 ml and reaches 256 000 g at 60 000 rpm.

Procedure: The plasma or lipoprotein samples were adjusted to an appropriate density (slightly higher than the heaviest part of the gradient) by addition of gradient material in solid state. Great caution was taken to add the material, especially sodium bromide, in small portions under moderate magnetic stirring to avoid temporary high concentrations of salt.

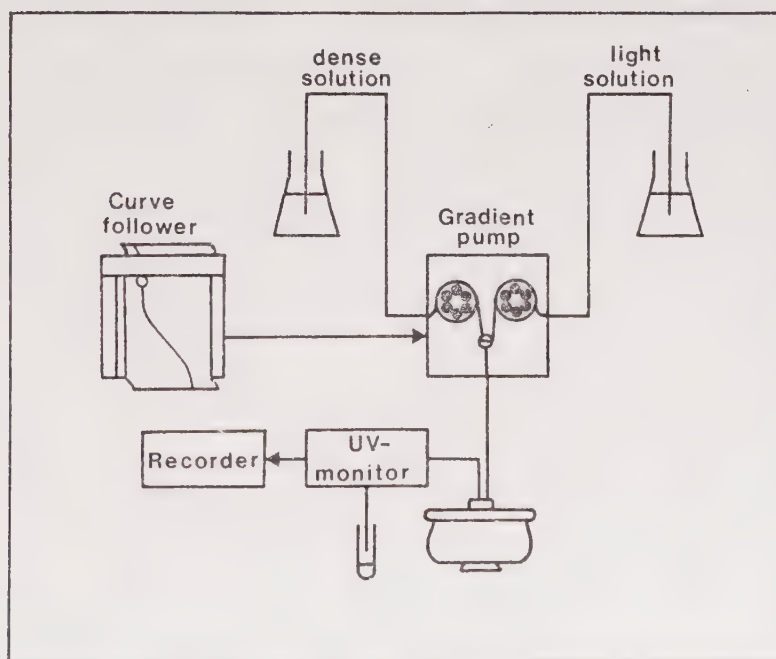


Fig. 1. Arrangement of equipment used in zonal ultracentrifugation. The pumps are used both for gradient formation and in the unloading procedure.

Unless otherwise stated, the centrifugations were carried out at $+15^{\circ}\text{C}$ and the solutions to be pumped into the rotor were thermostated at this temperature. In addition all solutions were passed through a heat exchanger before they reached the rotor. The Ti-14 rotors were run at 3 000 rpm and the Z-60 at 2 000-2 500 rpm while loaded and unloaded. With the Ti-14 rotor, the flow rate was kept at 20 ml/min during all steps, except for the sample loading which was accomplished at lower flow rates to avoid mixing. The gradient was introduced into the Z-60 rotor with 15 ml/min and all other steps were carried out at 10 ml/min.

Before starting the loading procedure the seal assembly and the flow lines were checked for possible leakage by introduction of 20 ml of the light solution used as overlay into the peripheral channels of the spinning rotor. This additional volume, which is eventually displaced from the rotor by introduction of the cushion, serves as a reserve in the case of minor cross leakages in the seal assembly or unforeseen slight deviations in the flow rate during loading. The loading procedure is then started by introduction of a calculated volume of a light solution (overlay) followed by the gradient starting with the light end.

After introduction of the sample the filling of the rotor was completed with a solution slightly denser than that of the sample (cushion). It is important to use a cushion volume (25-30 ml) large enough to ensure a complete displacement of the sample from the peripheral rotor wall.

After loading the rotor, the seal assembly was replaced by the rotor cap. Before closing the rotor chamber all accessible spillage and condensed water was thoroughly wiped off to secure a sufficient vacuum within a reasonable time. The rotor was accelerated to high speed (40 000-48 000 rpm for the Ti-14 and 55 000-60 000 rpm for the Z-60). After the appropriate centrifugation time, the speed was reduced to that used for the loading procedure. The seal assembly was again applied and 10-20 ml of light solution (extra overlay) was pumped into the centre of the rotor in order to prevent air present in the seal assembly to enter the rotor. The rotor was then centre-unloaded by introducing a solution of at least the same density as the cushion at the edge of the rotor.

The rotor effluent was passed through a UV-absorbance monitor (280 nm) and was then collected in 5-10 ml fractions. The UV-absorbance of the fractions was measured with a Zeiss spectrophotometer (PM6K). The density gradient was determined by weighing 5 ml aliquots of every fifth fraction, measured with a precisely calibrated pipette.

Running conditions for different types of separations in NaBr gradients are summarized in Table I.

TABLE I

Lipoprotein separations in sodium bromide gradients

Separation	Typical sample volume (ml)	Sample		Overlay		Gradient ^x		Cushion		Speed (rpm)	Run time (hr)
		density (kg/l)	volume (ml)	density (kg/l)	volume (ml)	density (kg/l)	volume (ml)	density (kg/l)	volume (ml)		
VLDL, large scale	400	1.16	75	150	1.00-1.15	40	1.40	44 000	0.5		
VLDL-IDL	25	1.16	60	550	1.00-1.15	30	1.20	48 000	0.5		
VLDL-LDL	25	1.16	10	600	1.00-1.15	30	1.20	48 000	1.5		
Main density classes	25	1.295	10	600	1.00-1.29	30	1.30	46 000	15-20		
HDL	25	1.395	10	600	1.00-1.39	30	1.40	44 000	20		
HDL-VHDL, large scale	250	1.25	75	{ 100 150 }		{ 1.10 1.20 }		75	1.40	44 000	48

^x All gradients used have been linear with respect to (rotor) volume.

RESULTS

Separation of the main lipoprotein density classes

Separation of VLDL, LDL, and HDL could be obtained in a single overnight run as seen in Fig. 2, which illustrates the ultracentrifugal pattern of 35 ml of human plasma. The first two peaks, showing immunoreactivity towards anti-B, consist of VLDL and LDL, respectively. The following two peaks represent HDL with immunoreactivity against anti-A-I but not anti-B. The HDL is also well separated from the bulk of plasma proteins as judged from the distributions of immunoreactivity towards anti-A-I and anti-albumin. Only trace amounts of A-I appeared in the plasma protein fraction. The resolution power remained essentially unchanged even when the plasma volume was increased to 75 ml.

Canine plasma lipoproteins could also be separated in the same way (Fig. 3). The canine lipoprotein pattern differed in some respects from the human pattern. Most notable is the separation of LDL into two distinct, partially resolved subclasses, which were also found in porcine plasma (14). On the other hand, porcine HDL could not be resolved into subclasses but appeared as a narrow, symmetrical peak in the zonal ultracentrifugation diagrams.

Separation of VLDL-LDL

Separations of VLDL and LDL from HDL and the bulk of plasma proteins were achieved in less than two hours with suitable gradients as shown in Fig. 4. The LDL peak was recovered at a density of about 1.09 kg/l, which means that isopycnic conditions were not reached in contrast to overnight runs (Fig. 2), where the peak was found at densities between those of VLDL and LDL (intermediate density lipoprotein or IDL).

Several patterns of IDL have been found. The detection of such particles in normal human plasma generally demands zonal ultracentrifugation of comparatively large plasma samples (100-150 ml) which does not seem to interfere with the resolution power. In fact large scale preparations of VLDL from 400 ml plasma are possible by a run for only 30 minutes as seen in Fig. 5, showing complete separation of VLDL from LDL with small amounts of intermediate density material appearing between these fractions.

In contrast with LDL from dog and pig, human LDL could not be separated into two subclasses. Only in a few individuals with distinct

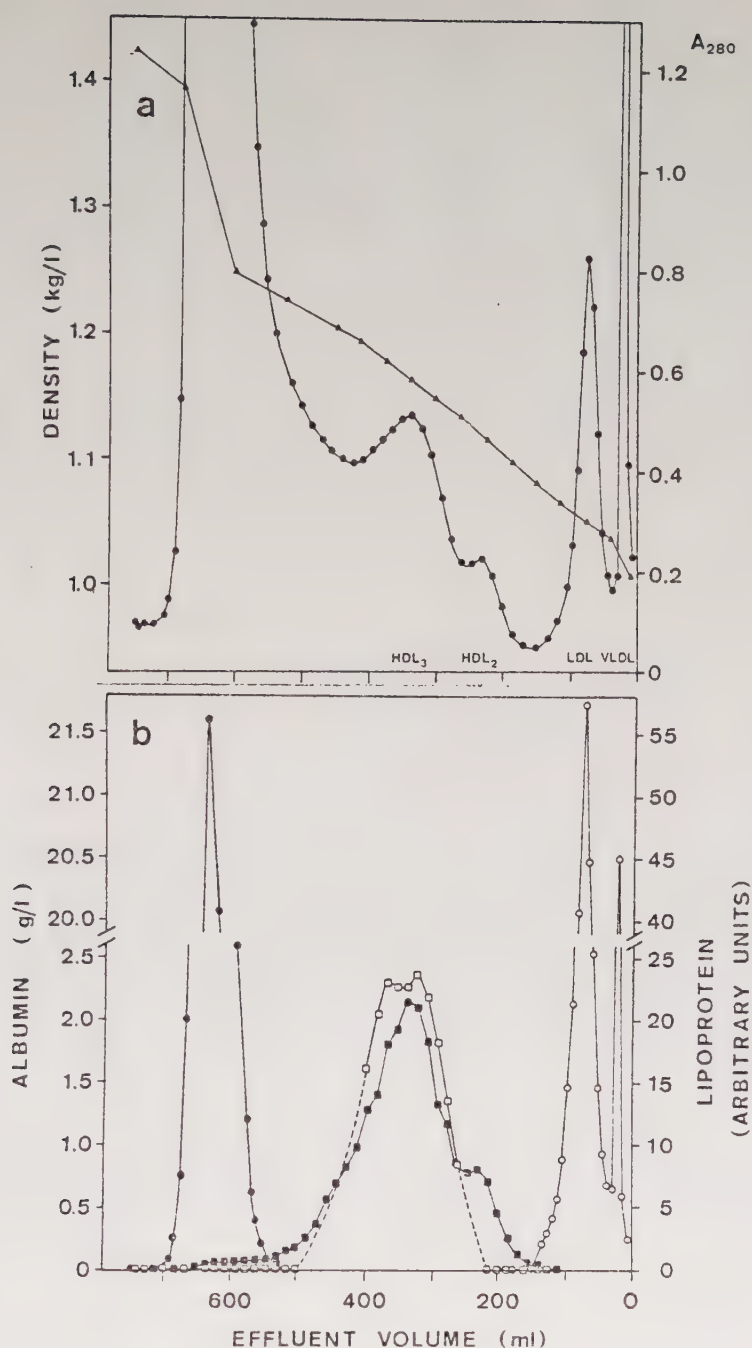


Fig. 2. Separation of the main density classes of lipoproteins from 35 ml normal human plasma. The Ti-14 rotor was run for 20 hours at 46 000 rpm at 15°C. Gradient: 600 ml NaBr solution, 1.00-1.29 kg/l, linear to rotor volume. Overlay: 15 ml buffer solution. Cushion: 30 ml NaBr solution, 1.30 kg/l. The upper part of the figure (a) shows the density gradient (▲—▲) after centrifugation and the absorbance at 280 nm of the fractions (●—●). The lower half of the figure (b) demonstrates the distributions of albumin (●—●), A-I (■—■), A-II (□—□), and B (○—○) determined with electroimmuno assay. The dashed line corresponding to A-II indicates the presence of faint ill-defined immunoprecipitates.

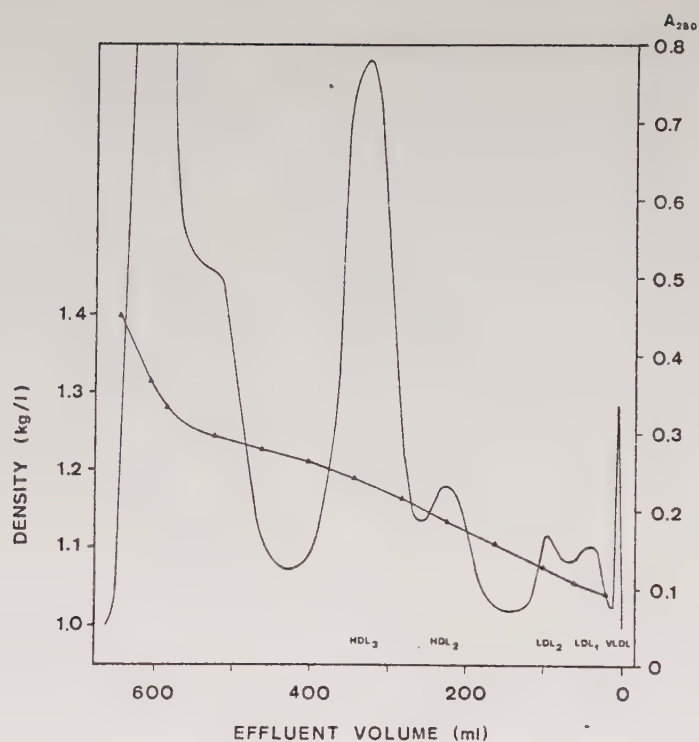


Fig. 3. Separation of the main density classes of lipoproteins from 25 ml canine plasma run for 15 hours at 46 000 rpm. Gradient: 600 ml NaBr solution, 1.00-1.29 kg/l, linear to rotor volume. Overlay: 10 ml buffer. Cushion: 30 ml NaBr, 1.30 kg/l. — Absorbance at 280 nm, continuously recorded. ▲—▲ Density gradient after centrifugation.

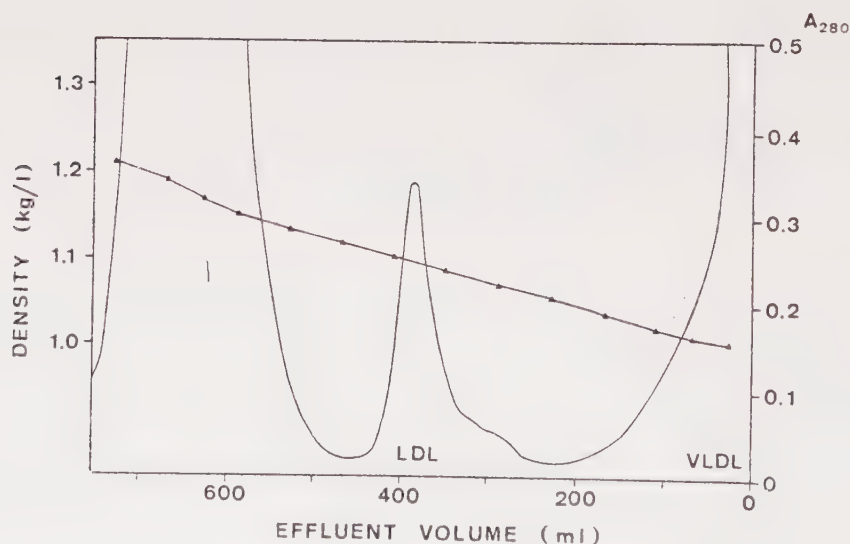


Fig. 4. Separation of VLDL and LDL from 20 ml normal human plasma run for 90 minutes at 48 000 rpm. Gradient: 600 ml NaBr, 1.00-1.15 kg/l, linear to rotor volume. Overlay: 15 ml buffer solution. Cushion: 30 ml NaBr solution, 1.20 kg/l. — Absorbance at 280 nm. ▲—▲ Density.

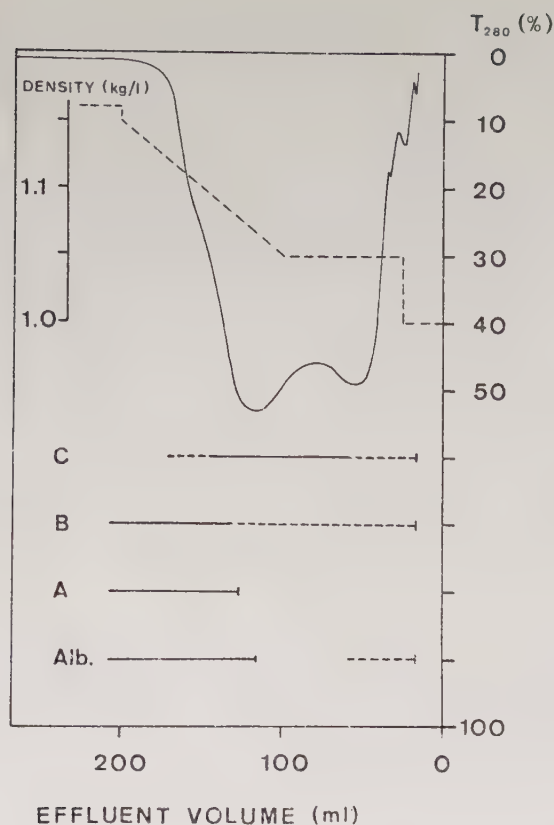


Fig. 5. Large scale separation of VLDL from a 400 ml sample of normal human plasma from a single donor. The rotor was filled in the following way: 30 ml buffer solution; 70 ml NaBr solution, 1.05 kg/l; 100 ml NaBr gradient, 1.05-1.15 kg/l; 400 ml sample with the density adjusted to 1.16 kg/l. As cushion was used 50 ml NaBr solution, 1.40 kg/l. The sample was run for 30 minutes at 46 000 rpm. ——— Transmission (%) at 280 nm. ----- Initial density distribution. The immunochemical presence of apo C (C), LpB (B), apo A-I (A), and albumin (Alb) is indicated in the lower part of the figure.

bands of pre- β_1 -lipoprotein (24) present on lipoprotein electrophoresis in agarose gel, an LDL pattern characterized by a partial resolution into two components was obtained which was most distinct on zonal ultracentrifugation for 4 hours under the conditions given in Fig. 6. One of these components (left in the figure) is probably identical with the so-called sinking pre- β -lipoprotein carrying the Lp(a)-antigen (25). The localization of the components in the gradient (around 1.10 kg/l) indicates that a zonal rate rather than an isopycnic separation was obtained in this case.

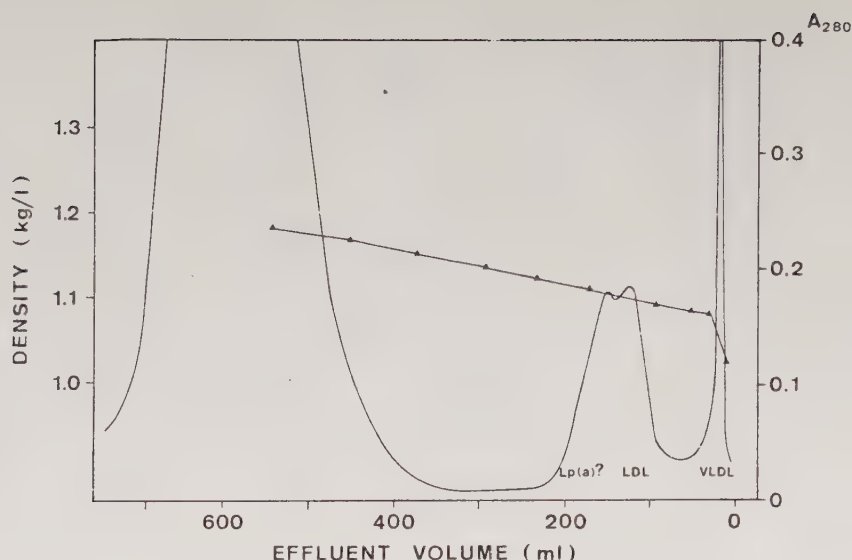


Fig. 6. Separation of VLDL and LDL from plasma of a healthy individual with a marked pre- β_1 -lipoprotein fraction (24) on electrophoresis in agarose gel. Separation of 25 ml plasma was performed for 4 hours at 48 000 rpm. Gradient: 600 ml NaBr, 1.08-1.19 kg/l, linear to rotor volume. Overlay: 15 ml buffer solution. Cushion: 30 ml NaBr solution, 1.20 kg/l. — Absorbance at 280 nm. $\blacktriangle$ — $\blacktriangle$ Density.

Separation of HDL-VHDL

The procedure used for the separations of the three main lipoprotein classes (VLDL, LDL, HDL) gave a separation of human and canine HDL into fractions corresponding to HDL₂ and HDL₃ (Figs. 2 and 3), with a running time of only 15-20 hours. The resolution of HDL₂ and HDL₃ could be varied to some extent by changes in the shape of the gradient. The proportions of HDL₂ and HDL₃ varied considerably in different pathological conditions as exemplified in Fig. 7, showing the remarkable HDL pattern of plasma from a patient with a recent debauch of heavy alcohol consumption.

The separations of HDL subclasses under standard conditions (Table I) were very reproducible. The HDL₂ and HDL₃ peaks were localized at densities of 1.115 ± 0.004 kg/l and 1.155 ± 0.006 kg/l, respectively (mean $\pm$ SD, $n = 10$). When recentrifuged under the same conditions both HDL₂ and HDL₃ appeared homogeneous and were recovered at their original densities (Fig. 8). Minor amounts of material appeared at higher densities.

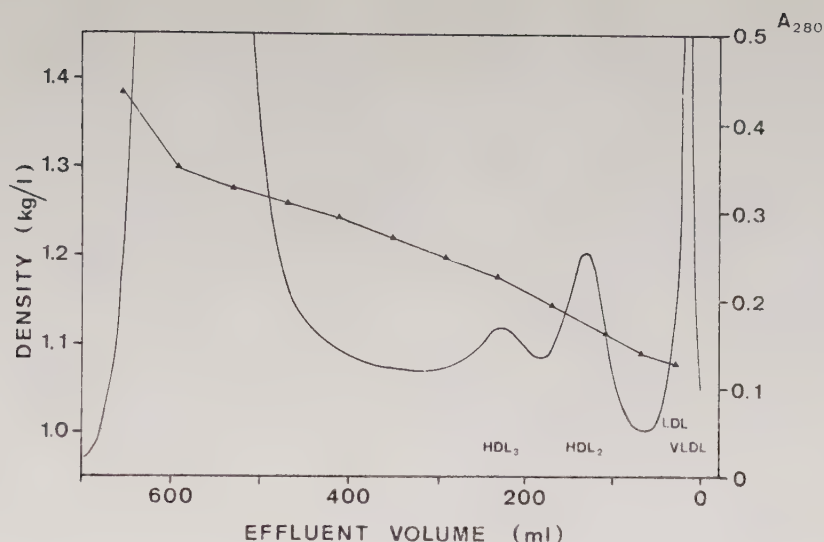


Fig. 7. Distribution of HDL in plasma from a male alcoholic. Separation of 20 ml for 20 hours at 46 000 rpm. Gradient: 550 ml NaBr solution, 1.00-1.29 kg/l. Overlay: 50 ml buffer solution. Cushion: 45 ml NaBr solution, 1.30 kg/l.
 — Absorbance at 280 nm. ▲—▲ Density.

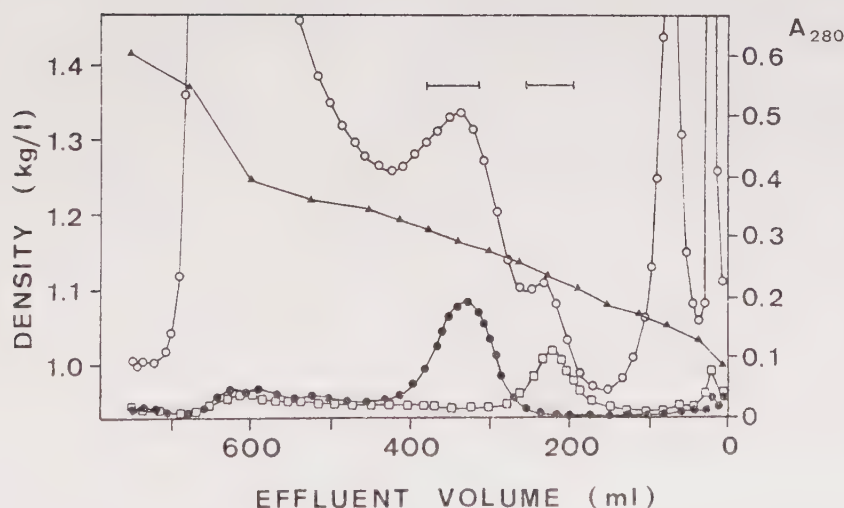


Fig. 8. Recentrifugation of HDL₂ and HDL₃ obtained from 35 ml normal human plasma. For experimental conditions, see Fig. 2. ○—○ Absorbance at 280 nm from the first run. Absorbance at 280 nm from the separate recentrifugations of HDL₂ (□—□) and HDL₃ (●—●) fractions, which were pooled as indicated by the horizontal bars.

The subclasses HDL₂ and HDL₃ separated by zonal ultracentrifugation had principally the same lipid and apoprotein composition as the corresponding fractions obtained by differential centrifugation, with one important exception. The proportion of apo A-II to apo A-I was considerably lower in the HDL₂ separated by zonal ultracentrifugation. Only small amounts of apo A-II were found in this fraction as judged from the electrophoretic patterns in polyacrylamide gels.

The minute amounts of material seen between LDL and HDL₂ ("HDL₁") had an apoprotein composition resembling HDL₂, sometimes with a slight contamination of LpB. A distinct component, however, regularly appeared in this density region when plasma from patients with obstructive liver disease was centrifuged. This component, tentatively called HDL₁-C (cholestatic HDL₁), has been shown to have a unique lipid and protein composition, dominated by phospholipid, unesterified cholesterol and the arginine-rich protein or apo E (12).

The VHDL part of the lipoprotein spectrum can be studied by extended runs for 36-48 hours. In these experiments LDL and VLDL were recovered in one peak and HDL, partially resolved into HDL₂ and HDL₃, was separated from the bulk of plasma proteins by a broad zone containing VHDL (Fig. 9). Agarose gel electrophoresis of fractions from this zone revealed lipoproteins of approximately the same mobility as the main HDL. A distinct component with a slower mobility similar to that of α LpB (26) was an inconstant finding. Apoprotein A-I completely dominated the protein part, with only trace amounts of apo C and apo A-II visible.

Determinations of the LCAT activity of the centrifugation fractions clearly demonstrated that this enzymatic activity was localized in the VHDL (Fig. 9). The VHDL can accordingly serve as a suitable source for the purification of LCAT from plasma on a 200-300 ml scale.

DISCUSSION

Although other separation procedures have been described for the isolation of plasma lipoproteins (27, 28) preparative ultracentrifugation is still the most widely employed method. The use of density gradient centrifugation in zonal rotors has several definite advantages over the conventionally used differential ultracentrifugation at fixed densities. While sequential differential centrifugation for the isolation of the three main density classes (VLDL, LDL, HDL) requires repeated centrifugations

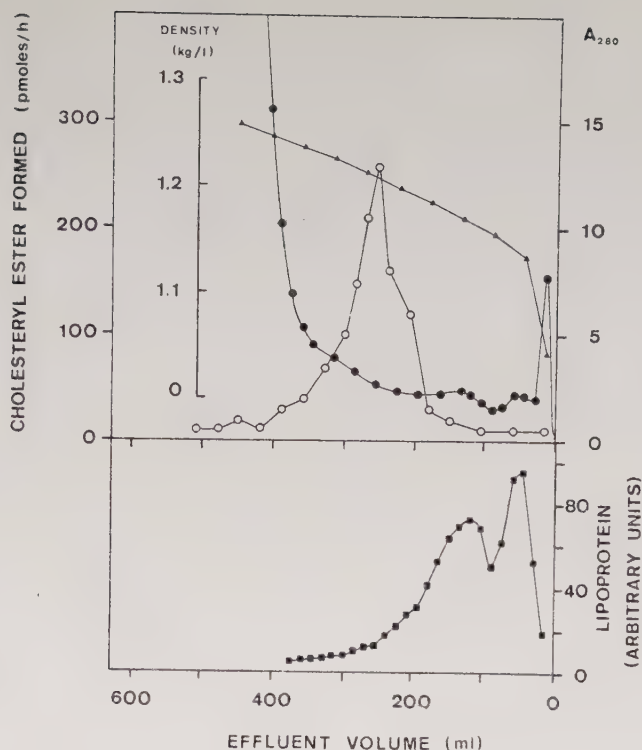


Fig. 9. Separation of HDL-VHDL by extended centrifugation of 250 ml normal plasma treated with dithiobisnitrobenzoic acid (Ellman's reagent), 1.5 mmol/l final concentration. The rotor was loaded with NaBr solutions of stepwise increasing concentrations: I, 75 ml, 1.00 kg/l; II, 100 ml, 1.10 kg/l; III, 150 ml, 1.20 kg/l; IV, sample, 1.25 kg/l; V, 75 ml, 1.40 kg/l. This run was performed for 48 hours at 44 000 rpm and at +5°C. The upper part of the figure shows absorbance at 280 nm (●—●), density (▲—▲), and LCAT activity (○—○). The lower part of the figure shows the distribution of apo A-I (■—■) determined with electro-immuno assay.

during more than one week with possible risks for alteration of lipoproteins, such a separation can be performed in less than 24 hours with zonal ultracentrifugation. Earlier described procedures have generally involved two-step procedures with change of gradients during the run (6, 9), but the present work shows that it is possible to obtain a satisfactory separation in a single, uninterrupted run during 15 hours.

A further advantage over the differential ultracentrifugation is the continuous distribution of lipoprotein material over a continuous density interval without predetermined density limits. Lipoproteins in the VLDL-LDL region can be separated in several populations by a single one hour run, employing comparatively large plasma samples essentially

without loss of resolution (cf. ref. 29 using differential centrifugation for this purpose). This separation procedure seems useful for several purposes, e.g. in the large scale preparation of VLDL, for studies of the VLDL-LDL distribution in patients with hyperlipoproteinemia (11) and in studies of the sequential lipolytic degradation of VLDL.

The fixed density limits in differential centrifugation may give incomplete information on the lipoprotein distribution in studies of animal lipoproteins or in human pathological conditions. Porcine and canine LDL is evidently composed of two subclasses (15-17) not discerned in previous work using preparative ultracentrifugation (30). The density distribution of HDL also differs between various species (14-16). The appearance of an abnormal HDL₁ in cholestasis in man was readily detected by zonal ultracentrifugation (12) which also allows isolation of the component in a single step for further studies.

The resolution power of zonal ultracentrifugation of HDL is not confined to separations of HDL₂ and HDL₃. With the application of higher centrifugal forces possible with the Z-60 rotor three distinctly separated subclasses could be obtained (31).

A potential drawback of the zonal ultracentrifugation procedure might be the exposure of lipoproteins to salt concentrations higher than those used in the differential ultracentrifugation technique. Although high concentrations of sodium bromide have been shown to accelerate transfers of apoproteins between HDL₂ and HDL₃ (32), the work of Patsch et al. (10) did not reveal any harmful effects to HDL during its isolation with zonal ultracentrifugation. Only small amounts of immunoreactive apo A-I was found in the plasma protein fraction in the zonal ultracentrifugation experiments presented in this work even when recentrifugation was undertaken in contrast to differential ultracentrifugation where the losses may amount to 10-50% during centrifugation and recentrifugation (33, 34). In addition the apoprotein compositions of the HDL₂ subclass, isolated by zonal ultracentrifugation and differential ultracentrifugation, differ markedly. The zonal ultracentrifugation procedure gives an HDL₂ with a very low content of apo A-II as demonstrated by Kostner et al. (9) and confirmed in the present work. The reason for this discrepancy is so far unknown.

It should be possible to use non-ionic gradient media in order to avoid high ionic strengths. Preliminary work in our laboratory has shown that D₂O gradients give essentially the same separations of VLDL-IDL-LDL as gradients of sodium bromide. Due to the limited density interval which can be obtained with D₂O, metrizamide [®] or sucrose gradients were

used for the separation of HDL. The most satisfactory results were obtained with metrizamide. The three main lipoprotein classes were well resolved and separated from the bulk of plasma proteins. Although the separation of HDL into HDL₂ and HDL₃ so far has been rather poor it could be shown that the lightest HDL particles corresponding to HDL₂ had apparent low content of apo A-II in relation to apo A-I (unpublished results). This observation indicates that the apoprotein composition of HDL₂ separated in sodium bromide gradients is not due to derangement of HDL₂ by the high salt concentration. Further experiments are needed to substantiate these findings.

It should be noted that both metrizamide and D₂O are expensive and furthermore the absorption of ultraviolet light by metrizamide is inconvenient, since it precludes the use of ultraviolet spectrophotometry as a very simple method for recording lipoprotein patterns after zonal centrifugation.

A further drawback of the gradient centrifugation method employing zonal rotors is that only one sample can be processed in the rotor, which is a disadvantage in for example clinical studies. In such instances swing out rotors can be used, preferentially for plasma samples not exceeding 0.5 ml with a 6 x 14 ml rotor or 2 ml with a 6 x 38 ml rotor. Methods for purifications of lipoproteins on a small scale have recently been described (35, 36), which give almost the same resolutions as that obtained in the special zonal rotors.

ACKNOWLEDGEMENTS

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Immunochemical Studies of Lipoproteins in Cholestasis

R. EKMAN, B. G. JOHANSSON & B.-G. PETERSSON

Department of Clinical Chemistry, University Hospital,
Lund, Sweden

Ekman, R., Johansson, B. G. & Petersson, B.-G. Immunochemical Studies of Lipoproteins in Cholestasis. *Scand. J. Immunol.* 4, Suppl. 2, 13-18, 1975.

Lipoproteins in normal and cholestatic human plasma were studied using crossed immunoelectrophoresis and electroimmunoassay. Crossed immunoelectrophoresis against anti- α -lipoprotein revealed a complex pattern of α -lipoproteins in cholestasis resembling that of α -lipoproteins in a patient with familial deficiency of the enzyme lecithin cholesterol acyl transferase. With anti- β -lipoprotein an absence of pre- β -lipoproteins with normal electrophoretic mobility was noted in crossed immunoelectrophoresis. These observations indicate that false values may be obtained with the use of electroimmunoassay for the determination of lipoprotein concentrations in cholestasis. A method employing crossed immunoelectrophoresis with an intermediate gel for the quantitative determination of lipoprotein X is proposed.

R. Ekman, M.D., Dept. of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden

Pronounced changes of the plasma lipoprotein composition are common in obstructive jaundice with an increased concentration of low-density lipoproteins (LDL) and a decreased concentration of high-density lipoproteins (HDL). The increase in the LDL fraction is at least in part due to the occurrence of an abnormal lipoprotein called lipoprotein X (LpX) which has a high content of unesterified cholesterol and phospholipids and is immunologically different from normal LDL (15). LpX was shown to have a peculiar protein composition, consisting of C apoproteins and albumin but no apoprotein B. The decrease in HDL is also most probably accompanied by changes in its apoprotein composition (13, 16).

Immunochemical studies of lipoproteins have commonly been performed with double-diffusion techniques or classical immunoelectrophoresis. The development of new high resolving techniques, e.g. crossed immunoelectrophoresis (11), can be expected to allow more detailed studies of the electrophoretic distribution and antigenic complexity of normal and pathological lipoproteins. It would be advis-

able to carry out such studies before attempts are made to determine lipoproteins with quantitative immunochemical methods such as single radial immunodiffusion (12) or electroimmunoassay (10).

This paper presents the results of an immunochemical study of lipoproteins in human cholestasis by means of electroimmunoassay and crossed immunoelectrophoresis. It is concluded that the results of quantitative immunochemical determinations of these lipoproteins by electroimmunoassay must be interpreted with great caution, due to the marked changes in their immunological behaviour as demonstrated with crossed immunoelectrophoresis.

MATERIALS AND METHODS

Blood samples were obtained from patients with extrahepatic biliary obstruction and from apparently healthy volunteers. The diagnoses in the patients were established by standard laboratory tests, liver biopsy, and surgical procedures. Plasma deficient in lecithin cholesterol

acyl transferase (LCAT) was obtained from a patient with a familial LCAT deficiency (6).

Preparation of lipoproteins.* Human LDL and HDL were prepared by differential flotation in the ultracentrifuge as described by Havel et al. (7). Lipoprotein X was prepared by a combination of polyanion precipitation (15) and immunoabsorption of contaminating lipoprotein B (2, 18) according to the following schedule: 1) precipitation of the low-density lipoproteins with heparin-MnCl₂; 2) immuno-precipitation of apo-B containing lipoproteins with an immunoglobulin preparation of rabbit anti-human β -lipoprotein; and 3) flotation of lipoprotein X for 24 hrs at a density of 1,063 g/cm³. The purity of the lipoprotein preparations was checked with agarose gel electrophoresis and staining with sudan black B and Coomassie brilliant blue (8).

Production of antisera. Antisera against HDL, LDL, and lipoprotein X were raised in rabbits by intramuscular injections of 0.2–0.5 mg of purified lipoproteins emulsified with complete Freund's adjuvant. The injections were repeated after 3 and 6 weeks. Satisfactory antibody titres were generally obtained 2 weeks after the last injections. The antisera were absorbed in order to render them monospecific. Anti- α -lipoprotein was obtained by absorption of anti-HDL with the infranant fraction after ultracentrifugation of normal serum at a density of 1.25 g/cm³ for 72 hours and with pure normal LDL. Anti- β -lipoprotein was obtained from anti-LDL by absorption with the infranant fraction of normal serum centrifuged at 1,063 g/cm³ for the preparation

of β -lipoprotein. Anti-LpX was also absorbed with this fraction as well as with pure normal LDL. One of the anti- α -lipoprotein sera still contained small amounts of anti- α_1 -antitrypsin antibodies after absorption. Preparation of the immunoglobulin fraction of the antisera was performed according to Steinbuch & Audran (17).

Lipoprotein electrophoresis was performed in agarose gel (8).

Electroimmunoassay was performed according to Laurell (10). α -lipoprotein analyses were run for 3 hours at 15 volts/cm, whereas β -lipoproteins were run overnight (16 hours) at 8 volts/cm.

Crossed immunoelectrophoresis was carried out according to the directions given by Ganrot (4).

Crossed immunoelectrophoresis with an intermediate gel was performed as described by Axelsen (1).

RESULTS

Studies with anti- α -lipoprotein. Electroimmunoassay of α -lipoprotein in normal plasma under the conditions described gave distinct immunoprecipitates, which were visible after staining with sudan black (Fig. 1A). The im-

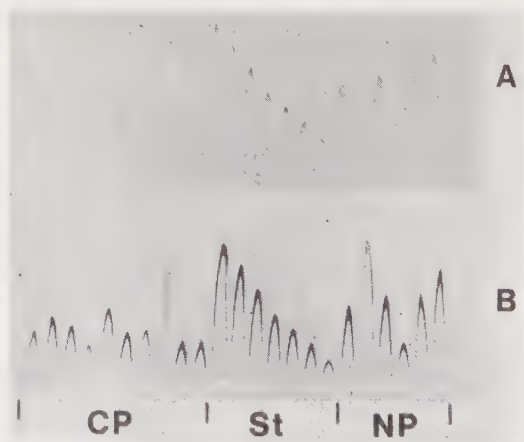


Fig. 1. Electroimmunoassay of α -lipoprotein in plasma. The plate was stained with sudan black (A) and with Coomassie brilliant blue (B). The precipitates were formed in cholestatic plasma (CP) and in normal plasma (NP). A serial dilution of normal plasma is used as standard (St).

* In this paper the designations VLDL (very low-density lipoprotein), LDL (low-density lipoprotein), and HDL (high-density lipoprotein) refer to fractions obtained by preparative ultracentrifugation, whereas α -, β -, and pre- β -lipoproteins are designations for electrophoretic fractions. Lipoprotein X (LpX) is used for the abnormal lipoprotein occurring in cholestasis. After absorption the antiserum obtained by immunization with HDL (anti- α -lipoprotein) contains antibodies to the A apoproteins and the antiserum against LDL (anti- β -lipoprotein) contains antibodies to apoprotein B, whereas anti-lipoprotein X contains antibodies to the C apoproteins.

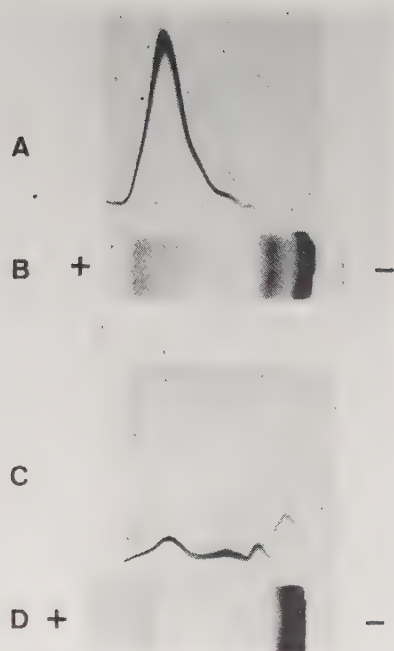


Fig. 2. Crossed immunoelectrophoresis of plasma against anti- α -lipoprotein. A. Normal plasma. C. Plasma from a patient with biliary obstruction. B and D are lipoprotein electrophoretic reference patterns. The faint precipitate seen in A and C is due to a slight contamination of anti- α_1 -antitrypsin. In C the cathodal part of the faint stainable precipitate is thought to be a complex of α_1 -antitrypsin and IgA.

munoprecipitates formed by the human plasma of patients who had suffered from obstructive jaundice from more than 2 weeks were almost invisible after staining with sudan black. After staining with Coomassie brilliant blue no difference was seen between normal and cholestatic plasma except in one case (Fig. 1B).

The electrophoretic pattern of plasma α -lipoprotein in cholestasis was quite different from that of normal plasma, as shown by lipoprotein electrophoresis and by crossed immunoelectrophoresis. Only one of the two normal bands in the α -lipoprotein region could be seen in cholestatic plasma (Fig. 2B and D). This remaining band presumably corresponds to serum albumin (3). Crossed immunoelectrophoresis revealed the presence of material immunologically related to normal α -lipopro-

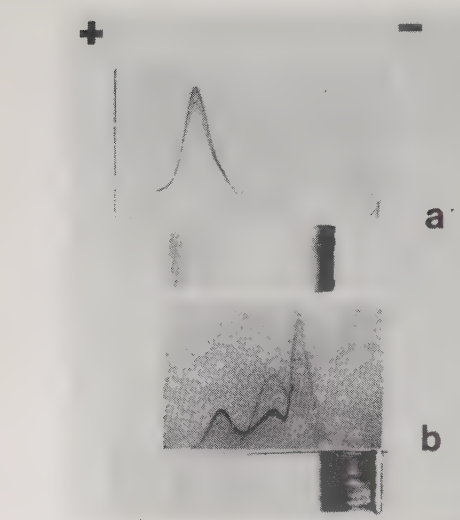


Fig. 3. Crossed immunoelectrophoresis of plasma against anti- α -lipoprotein. a) Normal. b) Familial lecithin cholesterol acyl transferase deficiency.

tein, but with different electrophoretic distribution (Fig. 2A and C). Instead of the single somewhat broad and asymmetrical peak from normal plasma, several peaks were found. The component with the most anodal position stained faintly with sudan black, whereas the others could be visualized only by staining with Coomassie brilliant blue. It should be noted,

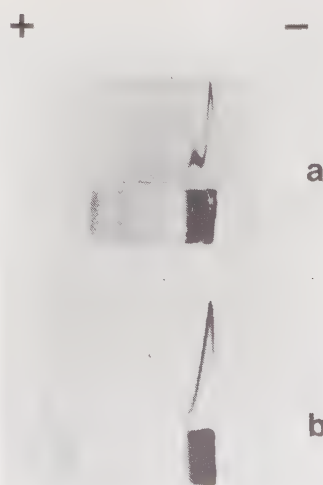


Fig. 4. Crossed immunoelectrophoresis of β -lipoprotein from a) normal plasma and b) cholestatic plasma.

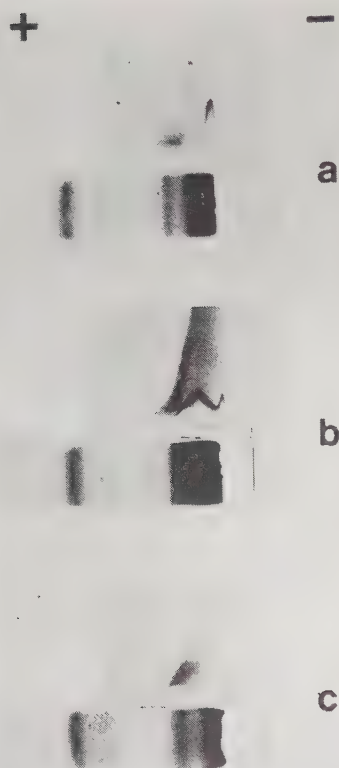


Fig. 5. Lipoprotein electrophoresis and crossed immunoelectrophoresis of plasma against anti-lipoprotein X. a) and b) Plasma from two patients with biliary obstruction. c) Normal plasma.

however, that a considerable variation in the patterns of cholestatic plasma was found. The extreme pattern illustrated here was only found in cases with cholestasis of a very long duration. For comparison the crossed immunoelectrophoretic pattern of plasma from a patient with familial LCAT deficiency is shown in Fig. 3. This condition is also characterized by the absence of an α -lipoprotein zone on lipoprotein electrophoresis. It is evident that the immunoprecipitation patterns of the LCAT-deficient plasma and cholestatic plasma have some common features as regards the electrophoretic distribution, but the pattern of LCAT-deficient plasma is more complicated.

Studies on β -lipoproteins (LDL). Electroimmunoassay of cholestatic plasma with anti- β -lipoprotein revealed no qualitative differ-

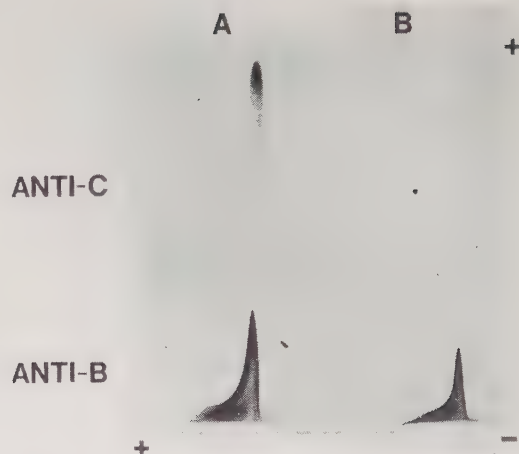


Fig. 6. Demonstration of lipoprotein X in crossed immunoelectrophoresis with intermediate gel. Cholestatic (A) and normal (B) plasma were used in the first dimension. The intermediate gel contained anti- β -lipoprotein (anti-B) and the upper gel anti-lipoprotein X (anti-C).

ences in the appearances of the immunoprecipitates compared to normal plasma. Crossed immunoelectrophoresis of plasma against anti- β -lipoproteins, on the other hand, revealed distinctly different patterns (Fig. 4). Normal plasma gave 2 well resolved peaks apparently corresponding to β -lipoprotein (LDL) and pre- β -lipoprotein (VLDL), respectively, whereas no distinct pre- β -lipoprotein peak was seen in cholestatic plasma. The lack of the normal pre- β -lipoprotein was also quite evident on lipoprotein electrophoresis of cholestatic plasma (cf. Fig. 4b). This absence of a pre- β -lipoprotein zone was a common finding in cholestasis.

In contrast to the distinct immunoprecipitates formed by β -lipoproteins, no well-defined rockets were found on electroimmunoassay of cholestatic plasma with anti-lipoprotein X. On crossed immunoelectrophoresis, however, a more distinct peak was formed with this antiserum. Figure 5 (a and b) shows the crossed immunoelectrophoretic pattern of cholestatic plasma with distinct immunoprecipitates, occupying the same positions on agarose gel electrophoresis as isolated lipoprotein X (3). These immunoprecipitates were also formed on crossed immunoelectrophoresis of lipo-

proteins recovered between 1,006 and 1,063 g/cm³ of cholestatic plasma, but absent from the lipoprotein fraction with a density of less than 1,006 g/cm³. In addition to the narrow peak in the β region one of the samples (Fig. 5b) showed a precipitate in the pre- β -lipoprotein region. A similar precipitate was also found in normal plasma, and most probably represents normal pre- β -lipoprotein (VLDL) (Fig. 5c).

In order to obtain a more specific method for immunochemical analysis of lipoprotein X, crossed immunoelectrophoresis with an intermediate gel (1) was employed. The antibodies against apoprotein B (anti- β -lipoprotein) constitute a barrier to lipoproteins containing both apoproteins B and C, e.g. VLDL. For this reason only lipoprotein X, containing apoprotein C but not apoprotein B, will reach the upper gel containing antibodies to C-apoproteins (Fig. 6). This technique should make it possible to determine lipoprotein X quantitatively.

DISCUSSION

Immunochemical analyses of the electrophoretic distribution of proteins by crossed immunoelectrophoresis give a more detailed picture than that obtained by conventional immunoelectrophoresis (cf. examples given in ref. 4). An abnormal distribution of plasma α -lipoproteins in cholestasis has been observed in earlier immunoelectrophoretic studies (13, 16) but the changes are more distinctly visualized by crossed immunoelectrophoresis. The abnormal α -lipoprotein components are poorly characterized but the poor staining ability with sudan black might indicate a relatively low content of lipids or an altered lipid composition. The cause of the α -lipoprotein changes is unknown. The resemblance between the α -lipoprotein pattern in cholestasis and that observed in the case of familial LCAT deficiency might indicate that a deficiency of this enzyme, which has also been observed in conditions with obstructive jaundice (5), plays an important role in the α -lipoprotein changes in cholestatic plasma. An impairment of the liver

function resulting in the synthesis of an altered apoprotein A could also contribute to the abnormal pattern (16).

Changes in the β -lipoprotein-pre- β -lipoprotein patterns in cholestasis are also easily demonstrated by crossed immunoelectrophoresis. The lack of a pre- β zone in many cholestatic plasma samples might be explained by the finding that the VLDL in cholestatic liver disease attains a β mobility (16). Another possible explanation might be that VLDL is decreased in cholestasis.

The marked changes of the lipoproteins make it difficult to perform accurate quantitative determinations of lipoproteins in cholestasis. This is true whether single radial immunodiffusion or electroimmunoassay is used. Crossed immunoelectrophoresis is a valuable aid in the interpretation of the results of immunochemical lipoprotein quantitation. The complicated antigenic composition of lipoproteins constitutes a further problem in immunochemical determination of lipoproteins. The occurrence of C apoproteins in VLDL as well as in lipoprotein X precludes attempts to determine the last-mentioned lipoprotein with simple immunochemical methods, but the use of the intermediate gel technique should render such determinations possible. A similar approach to lipoprotein X determination has recently been reported by Kostner (9).

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Characterization of high density lipoproteins in human cholestasis

B. ARNESJÖ, B. DANIELSSON, R. EKMAN, B. G. JOHANSSON & B. G. PETERSSON

Institute of Biochemistry, University of Lund, and Departments of Clinical Chemistry and Surgery, University Hospital, S-221 85 Lund, Sweden

Arnesjö, B., Danielsson, B., Ekman, R., Johansson, B.G. & Petersson, B.G. Characterization of high density lipoproteins in human cholestasis. *Scand. J. clin. Lab. Invest.* 37, 587-597, 1977.

High density lipoproteins (HDL) of plasma from patients with long-standing cholestasis were studied by several methods including crossed immunoelectrophoresis, gel filtration, and zonal ultracentrifugation. The crossed immunoelectrophoretic pattern against anti-HDL was characterized by the presence of several immunoprecipitates, in striking contrast to the pattern formed by normal HDL. One of these precipitates corresponded to a lipoprotein species dominated by apoprotein A-II (apo A-II). Zonal ultracentrifugation of cholestatic plasma showed a decrease of HDL and a disappearance of the normal delineation of HDL₂ and HDL₃. In addition a new component designated HDL₁-C was observed. Consistent results were found at studies of the molecular size distribution of cholestatic HDL by gel filtration on Sephadex G-200. The lipid and apoprotein composition of isolated cholestatic HDL fractions differed considerably from normal. Most notable were the high proportions of phospholipids and free cholesterol and the occurrence of the apoprotein E (arginine-rich protein) in remarkably high amounts especially in the abnormal HDL₁-C.

Key-words: apoprotein composition; arginine-rich protein; cholestasis; crossed immunoelectrophoresis; gel filtration; high density lipoproteins; lipid composition, zonal ultracentrifugation

B. G. Petersson, M.D., Department of Surgery, University Hospital, S-221 85 Lund, Sweden

Obstructive jaundice in man is commonly associated with profound changes in the plasma lipoproteins. The most well-known change is the appearance of an abnormal low density lipoprotein called lipoprotein-X (LP-X) [27, 28].

High density lipoproteins (HDL) also undergo changes in biliary obstruction. Most con-

spicuous is the finding of very low HDL levels, but the physico-chemical characteristics of cholestatic HDL are also markedly different from those of normal HDL, as judged by changes in molecular size distribution [5], and electrophoretic and immunoelectrophoretic patterns [8, 25, 29].

In a previous preliminary report zonal

TABLE I. Clinical data, results of liver function tests and immunologically estimated levels of α -lipoprotein in patients and healthy individuals

No.	Diagnosis	Sex	Age years	Duration of jaundice weeks	S-bilirubin $\mu\text{mol/l}$	S-Alkaline phosphatase $\mu\text{kat/l}$	S-ALAT $\mu\text{kat/l}$	α -lipo protein* %	Reaction against anti-HDL†	HDL ₁ -C	LP-X
1	Cancer pancreatis	M	80	3	277	45.1	2.40	43	3	+	+
2	Cancer pancreatis	M	57	2½	124	50.1	1.67	22	3	+	+
3	Cancer pancreatis	F	81	4	146	18.4	1.65	42	3	+	+
4	Cancer pancreatis	F	73	3	201	25.9	1.92	38	3	+	+
5	Cancer of the bile common duct	M	78	4½	139	37.7	2.64	37	3	+	+
6	Cancer of the bile common duct	M	78	3½	425	29.9	1.67	30	4	+	+
7	Cancer ventriculi with liver metastasis	M	58	3	408	7.2	0.35	41	3	+	+
8	Cancer ventriculi with liver metastasis	F	62	4	219	19.2	0.77	38	3	+	+
9	Reticulum cell sarcoma	M	34	3	133	25.1	1.30	40	3	+	+
10	Stone in the common bile duct	F	67	1	82	36.7	3.61	83	1	—	+
11	Stone in the common bile duct	M	63	3	185	24.1	2.45	52	3	+	+
12	Ulcerative colitis	M	55	—	12	4.7	0.44	66	1	—	—
13	Cancer ventriculi	M	75	—	7	4.6	0.32	62	1	—	—
14	Sarcoma retroperitonealis	M	58	—	6	5.0	0.51	83	1	—	—
15	Mc Crohn	M	30	—	7	6.1	1.02	64	1	—	—
16	Cancer ventriculi	M	74	—	12	4.4	0.33	54	1	—	—
17	Cancer ventriculi	F	68	—	3	2.2	0.29	79	1	—	—
18	Cancer coli	F	69	—	8	3.4	0.35	81	1	—	—
19	Myocardial infarction	M	68	—	14	4.1	0.65	79	1	—	—
20	Myocardial infarction	F	72	—	12	4.5	1.02	77	1	—	—
21	Myocardial infarction	M	59	—	16	3.8	0.86	70	1	—	—
22	Healthy	F	50	—	15.3	3.5	0.33	140	1	—	—
23	Healthy	M	35	—	10.2	2.7	0.38	110	1	—	—
24	Healthy	F	26	—	6.8	2.3	0.50	135	1	—	—
25	Healthy	F	25	—	10.2	2.8	0.30	94	1	—	—

* The amount of α -lipoprotein relative to that in pooled plasma from 80–100 male blood donors.

† The number of precipitates demonstrable with crossed immunoelectrophoresis.

ultracentrifugation patterns of cholestatic HDL were described [6]. It was found that material corresponding to HDL₂ and HDL₃ was greatly diminished while a new fraction appeared which was tentatively called HDL₁ on account of its position in the zonal ultracentrifuge pattern. The HDL₁ fraction had a lipid composition reminding of LP-X with comparatively high contents of unesterified cholesterol and phospholipids and had also a unique apoprotein composition.

The purpose of the present study was to characterize plasma HDL in biliary obstruction in terms of density distribution, electrophoretic mobility, particle size distribution and protein and lipid composition.

MATERIAL AND METHODS

Patients

Venous blood samples were obtained from eleven in-patients with biliary obstruction in association with various diseases (Table I), established by standard laboratory tests, liver biopsy, and surgical procedures. All patients were in sufficiently good general condition for laparotomy. The patients had been jaundiced for 2–5 weeks at blood sampling, except the patients with concrements in the common bile duct who had been jaundiced for only 1 week. Blood samples were also obtained from ten in-patients with severe diseases with inflammatory reactions and low α -lipoprotein but without biliary obstruction (Table I). Samples from four apparently healthy persons served as normal reference (Table I).

Blood samples were collected after the subjects had fasted for 14 h. Na₂EDTA (1 mg/ml blood) was used as anticoagulant. All samples were stored at +4°C and analysed within 24 h.

Preparations of lipoproteins and production of antiserum

Human HDL and low density lipoproteins (LDL) were prepared as described earlier [8]. The purity of the lipoprotein preparation was checked with agarose gel electrophoresis. The gels were stained with Sudan Black B and Coomassie Brilliant Blue R 250. The apoproteins A-I, A-II, and C-I were isolated by gel filtration

[10]. The purity of these apoproteins was controlled with urea-polyacrylamide gel electrophoresis. Antisera against HDL, LDL, and the apoproteins A-I, A-II, and C-I were raised in the same way as described earlier [8]. The antisera were made monospecific by appropriate absorption [8]. The antiserum against HDL contained antibodies against A-I and A-II but did not react with apo B or apo C. Antiserum against apo E was kindly supplied by Dr G. Kostner, Graz, Austria.

Isolation of HDL by zonal ultracentrifugation

Plasma samples (15–30 ml) were centrifuged in a Beckman Ti-14 zonal rotor at 44,000 rpm at +15°C for 20 h [6]. The pooled HDL-fractions were concentrated by membrane ultrafiltration (Millipore Corp., Bedford, Mass., U.S.A.) and dialysed against a buffer consisting of 0.02 mol/l Tris-HCl, pH 7.4, 0.001 mol/l Na₂EDTA, and 0.2 mol/l NaCl.

Gel filtration

Gel filtration on Sephadex G-200, superfine, particle size $20 \pm 2 \mu\text{m}$, screened by dry elutriation [9] was done at +4°C in 0.05 mol/l Tris-HCl, pH 7.4, containing 0.01 mol/l EDTA and 0.2 mol/l NaCl. Columns, $5 \times 90 \text{ cm}$, designed for upward-flow technique was used. The flow rate was kept at 12 ml/h. The effluent was collected in 9 ml fractions and the absorbance of each fraction was measured at 280 nm.

Analytical methods

Lipoprotein electrophoresis was performed in agarose gel as described by Johansson [17] and electroimmunoassay as described by Laurell [22]. The apparent amounts of HDL and LDL estimated by electroimmunoassay were expressed relative to those in pooled plasma from 80–100 apparently healthy male blood donors. Crossed immunoelectrophoresis was carried out according to Ganrot [11]. Crossed immunoelectrophoresis with an intermediate antibody-containing gel was performed as described by Axelsen [2]. Agar gel electrophoresis in Difco Bacto Agar with subsequent polyanion precipitation as described by Seidel, Wieland & Rupert [30] was used for the detection of LP-X.

Polyacrylamide gel electrophoresis

The apoproteins were separated by SDS-gel electrophoresis in polyacrylamide gels of 12% acrylamide concentration as described by King & Laemmli [21]. The samples were reduced by 0.1 mol/l 2-mercaptoethanol and heated in 0.1% SDS at 90°C for 90 sec. In some cases the reduction step was omitted. Polyacrylamide gel electrophoresis in 8 mol/l urea was performed as described by Davis [7]. The separations were carried out in a Pharmacia apparatus GF-4 (Pharmacia Fine Chemicals, Uppsala, Sweden) and the proteins were stained with Coomassie Brilliant Blue R-250.

Lipid analysis

Lipids were extracted in chloroform-methanol (2:1) and the lipid classes were separated by thin layer chromatography (TLC) on silica gel using petroleum ether-ethyl ether-acetic acid (90:10:1) as the mobile phase. The cholesterol and cholesterol ester were separated and eluted from the TLC plate with chloroform-methanol (8:1). The cholesterol content was determined according to Abell [1]. Phospholipids were determined as described by Bartlett [4] and triglycerides were measured by an automatic enzymatic method with reagents from Boehringer Mannheim.

RESULTS

Table I summarizes the clinical data, results of

the liver function tests and apparent levels of α -lipoprotein estimated by electroimmunoassay. In all jaundiced patients the α -lipoproteins were decreased. The electrophoretic patterns of lipoproteins from two patients who had had obstructive jaundice for 2 and 4 weeks and from a normal control are shown in Fig. 1. No band with normal α -lipoprotein mobility could be observed in these two cases. The only band in the α -region migrated like normal albumin.

Crossed immunoelectrophoresis of cholestatic plasma against anti-HDL also revealed marked changes compared to normal plasma as shown in Fig. 1. Instead of the broad asymmetrical peak in the α -region in the pattern of normal plasma, three or four peaks with different electrophoretic mobilities were found in plasma from patients with longstanding cholestasis (Fig. 1). The normal plasma samples were usually diluted 1/5 before electrophoresis in order to reduce the peak heights to the same levels as those obtained with undiluted cholestatic plasma, but even if the normal plasma was run undiluted no additional components were visible. These precipitates stained poorly with Sudan Black and they could be visualized only with protein stain (Coomassie Brilliant Blue), whereas the precipitates in the α -region in the same position as normal HDL took also lipid stain. The anti-HDL reacting components gave a continuous precipitation line with the exception of the additional component present in cases Nos 4, 5, 6, 7 and 8, and situated as shown in Fig. 1C.

Crossed immunoelectrophoresis with intermediate gels containing antiserum against

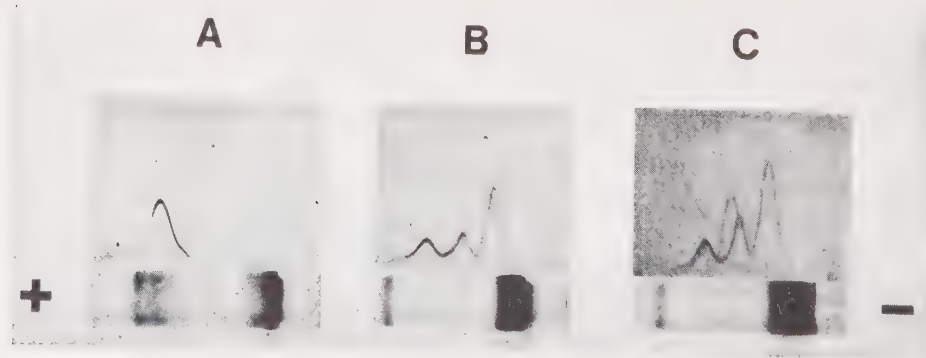


FIG. 1. Agarose gel electrophoresis and crossed immunoelectrophoresis against anti-HDL of plasma samples from (A) a normal individual, (B) patient No. 2, and (C) patient No. 6. The plasma sample in A was diluted 1/5 before electrophoresis.

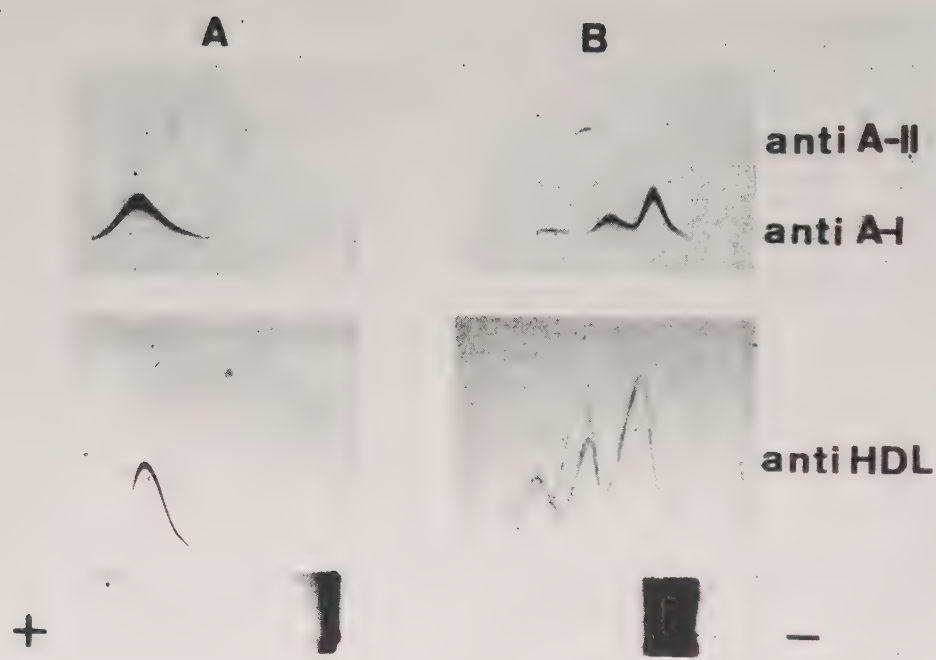


FIG. 2. Crossed immunoelectrophoresis against anti-HDL (below) and crossed immunoelectrophoresis with an intermediary antibody-containing gel run against specific antiserum towards apoproteins A-I and A-II applied as indicated in the figure (above). The samples were from (A) a healthy person, and (B) patient No. 6.

apoproteins A-I or A-II and with the complementary antiserum in the upper gel was used for further immunoelectrophoretic characterization of cholestatic HDL (Fig. 2). When anti-A-I was used in the intermediate gel, normal plasma

gave one distinct precipitate with anti-A-I and no material reacting with anti-A-II passed through this gel (Fig. 2A). The cholestatic plasma samples gave two or three distinct immunoprecipitates against anti-A-I, obviously

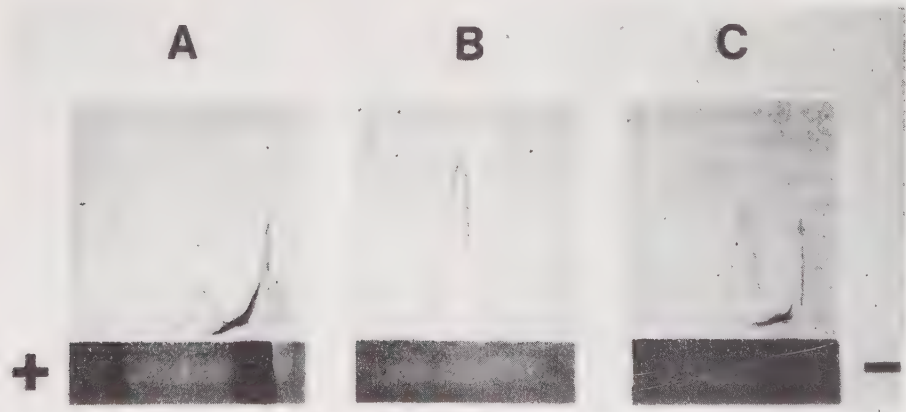


FIG. 3. Result of addition of cholestatic HDL₁ isolated by zonal ultracentrifugation to plasma from a patient with obstructive jaundice (No. 4). Crossed immunoelectrophoresis against anti-C of (A) cholestatic plasma, (B) pure HDL₁-C, and (C) cholestatic plasma with added HDL₁-C.

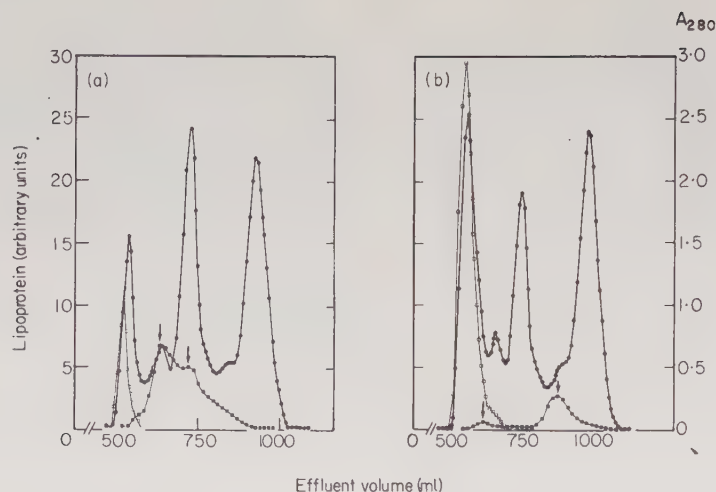


FIG. 4. Gel filtration experiments with plasma from (a) a normal female person (No. 24), and (b) a patient with obstructive jaundice (No. 4). 8 ml samples were applied to a 5×90 cm Sephadex G-200 superfine column and eluted with 0.05 mol/l Tris-HCl, pH 7.4, containing 0.2 mol/l NaCl and 0.01 mol/l EDTA. ●, A_{280} . □ and ■, electroimmunoassays with antiserum against human LDL and HDL, respectively. The arrows indicate fractions for which K_{av} values are given in Results.

corresponding to the continuous precipitates with several peaks found with anti-HDL (Fig. 2B). But, in contrast to what was found with normal plasma one precipitate was found also in the upper gel containing anti-A-II. The electrophoretic mobility of this precipitate was the same as that of the additional component diverging from the continuous line against anti-HDL. Consequently, this component contained A-II but very little or no immunoreactive A-I.

Additional immunochemical studies were performed in order to better characterize the most cathodical of the HDL components in cholestatic plasma. Crossed immunoelectrophoresis with an antiserum to apoprotein C-I gave the pattern seen in Fig. 3A. Besides the sharp peak, formed by lipoprotein-X and the partially resolved component corresponding to very low density lipoproteins (VLDL), a distinct peak was formed with the same localization as the cathodal precipitate against anti-HDL or anti-A-I. A peak in the same position was found for HDL₁-C isolated from cholestatic plasma [8] as shown in Fig. 3B. Addition of the pure HDL₁-C to the sample run in Fig. 3A resulted in an increase of the cathodal component without any other changes, indicating that this precipitate was formed by HDL₁-C (Fig. 3C).

Gel filtration

Gel filtration of plasma from healthy volunteers and from patients with long-standing cholestasis revealed rather striking differences in the size distribution of material reacting with anti-HDL. The HDL from normal plasma was almost completely separated from the LDL and appeared as a broad asymmetric peak containing several poorly resolved populations with K_{av} for the two main populations of 0.10 ± 0.01 and 0.19 ± 0.01 , respectively (Fig. 4a). The small fraction with comparatively large particle size present did not show any differences from the rest of the HDL in its crossed immunoelectrophoresis pattern against anti-HDL.

Gel filtration of plasma from the eleven patients with obstructive jaundice constantly gave a different pattern (Fig. 4b). In all but one case (No. 10) two completely resolved populations of anti-HDL reacting material were found with K_{av} values of 0.08 ± 0.01 and 0.32 ± 0.05 . Although these populations were well separated, small amounts of anti-HDL reacting material were found between them. The component eluted first showed only one peak, very faintly stainable with Sudan Black and situated in the position of HDL₁-C in crossed immunoelectro-

TABLE II. Lipid and protein composition of normal and cholestatic high density lipoproteins isolated by zonal ultracentrifugation. The fractions were pooled as indicated in Fig. 5A and 5B. The data are the mean values of three healthy individuals and five patients with obstructive icterus, ranges are given in parenthesis

Pooled fractions	Protein (%)	Lipid (%)	Triglycerides (%)	Cholesterol ester (%)	Unesterified cholesterol (%)	Phospholipids (%)
Normal						
HDL ₂	38.2 (34.4-42.9)	61.8 (57.1-65.6)	3.7 (2.8-5.2)	20.1 (16.5-23.6)	4.0 (2.8-5.5)	33.8 (32.0-35.8)
HDL ₃	49.8 (47.2-53.1)	50.2 (46.9-52.8)	2.4 (1.9-2.5)	15.3 (10.8-18.7)	2.5 (2.1-2.8)	29.9 (28.7-30.7)
Cholestatic						
HDL ₁ -C	30.4 (25.9-34.5)	69.6 (65.5-74.1)	4.0 (2.0-5.7)	5.4 (4.0-6.4)	11.5 (10.5-12.0)	48.5 (46.4-49.3)
HDL ₂ -C	42.5 (39.7-46.2)	57.5 (53.8-60.3)	4.3 (3.5-5.7)	8.4 (6.2-10.5)	7.0 (5.8-8.7)	37.5 (37.2-38.0)
HDL ₃ -C	49.3 (47.8-51.6)	50.7 (48.4-52.2)	2.2 (1.6-2.8)	4.9 (4.2-5.9)	3.7 (3.1-4.2)	40.0 (38.0-41.4)

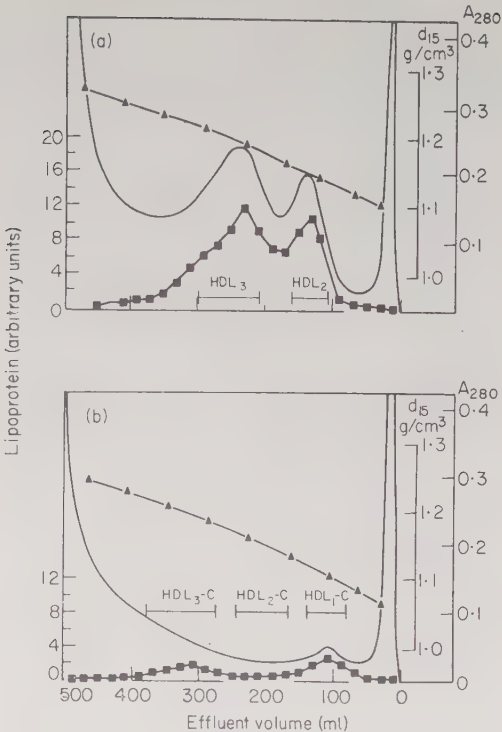


FIG. 5. Zonal ultracentrifugation in a discontinuous sodium bromide gradient of (a) 25 ml plasma from a normal female, and (b) 28 ml plasma from a patient with cholestasis. The rotor (Ti-14) was run 20 h at 44 000 rpm at 15°C. —, A₂₈₀; ▲, density at 15°C; and ■, electroimmunoassay against anti-HDL. Fractions were pooled as indicated in the figure.

phoresis against anti-HDL. The HDL-component eluted last behaved essentially like normal HDL₃ in crossed immunoelectrophoresis although the K_m value suggested a smaller molecular size than that of normal HDL₃.

Zonal ultracentrifugation

The zonal ultracentrifugal pattern of plasma from a healthy female is illustrated in Fig. 5a. There was a good resolution between HDL₂ and HDL₃ which were recovered at average densities of 1.15 g/cm³ and 1.19 g/cm³, respectively, with very little difference with sex. No contamination of the HDL fractions with albumin or LDL could be demonstrated immunochemically. Lipid and protein content (Table II) agreed well with data available in the literature for HDL₂ and HDL₃ isolated with other techniques [26] while the apoprotein

composition seemed to differ slightly with less apoprotein A-II in proportion to A-I than is generally found.

Zonal ultracentrifugation of plasma from patients with long-standing obstructive jaundice revealed very remarkable changes in the HDL patterns (Fig. 5b). The pattern was characterized by low amounts of HDL₂ and HDL₃ without the normal distinct demarcation of these two subclasses. In addition a new component had appeared at a lower density (HDL₁-C). This pattern was obtained in cholestasis with a duration of several weeks. The levels of HDL₁-C in such plasma were in the order of 0.5 g/l as judged from protein determinations and knowledge of the protein content of the lipoprotein.

The HDL fractions obtained by zonal ultracentrifugation of cholestatic plasma showed an apoprotein composition remarkably different from those of normal HDL fractions as illustrated in Fig. 6. The most striking change was the appearance of an apoprotein with an apparent molecular weight of 35,000 daltons in cholestatic HDL-fractions. This molecular weight suggested that the apoprotein was identical to apo E (arginine-rich protein) and this assumption was strengthened by the fact that HDL₁-C

gave a heavy immunoprecipitate in double diffusion tests against anti-apo E (kindly supplied by Dr G. Kostner, Graz, Austria). This apoprotein dominated the pattern of the cholestatic HDL₁-C, but was also abundant in the fraction obtained at a density corresponding to normal HDL₂. Cholestatic HDL₃, on the other hand, was more similar to its normal counterpart. Apoprotein A-I occurred in only small amounts in HDL₁-C, in about the same proportions as a low molecular weight component of about 8,500 daltons. The latter component was reasonably identical with A-II since it changed its position to about 17,000 daltons when the reduction step was omitted.

Polyacrylamide gel electrophoresis in 6 mol/l urea showed results in agreement with the SDS gel electrophoresis concerning apo A-I and apo A-II. Furthermore urea-polyacrylamide gel electrophoresis revealed the presence of considerable amounts of C apoproteins in the cholestatic HDL fractions. The abundance of apo C-I was notable especially in HDL₁-C.

Immunochemical studies revealed no apoprotein B in any HDL fraction from normal or cholestatic plasma. The stacking gels at SDS-polyacrylamide gel electrophoresis were also

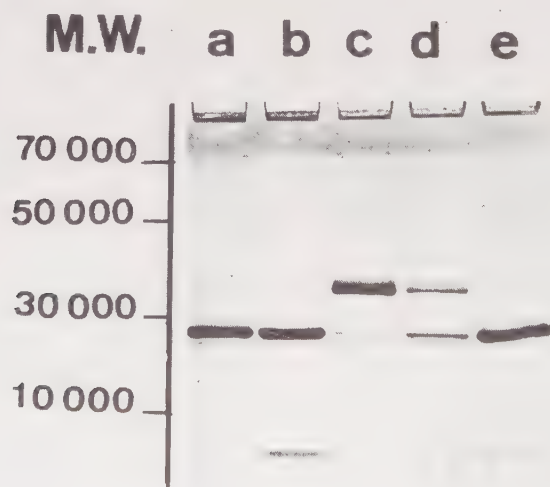


FIG. 6. Electrophoresis of apoprotein from isolated lipoprotein fractions in 12% polyacrylamide containing 0.1% SDS. All samples contained 15 µg protein and were reduced with 2-mercaptoethanol. The approximate molecular weight scale was deduced from the position of nine standard proteins. Normal lipoprotein samples: (a) HDL₂, and (b) HDL₃. Cholestatic lipoprotein samples: (c) HDL₁-C, (d) 'HDL₂-C', and (e) 'HDL₃-C'.

free from aggregated or high molecular weight material.

The lipid composition of cholestatic HDL fractions also differed considerably from that of normal plasma HDL (Table II). All cholestatic HDL fractions were relatively rich in phospholipids and showed a decreased cholesterol ester-cholesterol ratio, which was most pronounced in HDL₁-C. The data given in Table II is based on observations of patients jaundiced for 2-3 weeks. In some extreme cases HDL₁-C had only traces of cholesterol esters, the lipid part being totally dominated by free cholesterol and phospholipids.

Chronology and the effect of surgical treatment on the HDL pattern

The first change in cholestasis notable in crossed immunoelectrophoresis with anti-HDL was a decrease in HDL reflected by a diminished height of the precipitation peak. Generally two or three new additional components appeared after 2-4 weeks of obstructive jaundice.

Studies of three cases on repeated occasions with zonal ultra centrifugation indicated that the density distribution of lipoproteins in the HDL₂-region gradually shifted towards lower density with a concomitant decrease of material in this region. There was also a gradual decrease of HDL₃ and a change of the density distribution towards higher densities. A distinct HDL₁-C did not appear until the jaundice had lasted for at least 2 weeks. The situation illustrated in Fig. 5b appeared after several weeks.

In four cases the HDL patterns were registered on various occasions by crossed immunoelectrophoresis for 3 weeks after relief of their biliary obstruction by percutaneous transhepatic drainage. The component corresponding to HDL₁-C disappeared within the first week and within the following 1-2 weeks the HDL patterns were essentially normal. Principally the same results were obtained on repeated zonal ultracentrifugation in a chronologic study of two patients, i.e. the disappearance of HDL₁-C within one week and normalization of HDL₂ and HDL₃ within about 3 weeks. The decrease of the HDL₂ seemed to be the most persistent change.

DISCUSSION

The HDL metabolism is affected in many

physiological and pathological conditions. The plasma HDL levels are higher in females than in males owing to an increase especially in HDL₂ [3]. This is at least partly dependent on the influence of oestrogen on the metabolism of HDL. High HDL-levels are thus found in pregnant women [14] and in women taking oral contraceptives. Raised HDL-levels is a common finding in alcoholics after alcoholic débauches [19] and can also be seen in maturity onset diabetes [33]. On the other hand, somewhat low levels have been found in inflammatory conditions accompanied by acute phase reactions in the plasma protein patterns [17].

But more drastic changes of the HDL levels occur in cholestatic conditions [5, 6, 25, 29] and in the early icteric phase of viral hepatitis [20]. These changes are readily demonstrated by agarose gel electrophoresis which shows an apparent lack of the α -lipoprotein zone staining with Sudan Black [8] and by the changed appearance of the immunoprecipitates obtained at electroimmunoassay of α -lipoprotein or HDL.

Also crossed immunoelectrophoresis reveals marked changes of HDL in cholestasis as reported earlier [8] and in the present paper. All components corresponding to the several immunoprecipitates found have not as yet been characterized but it seems clear that one of them is formed by the abnormal HDL₁-C [6] and that one of the precipitates contains little or no apo A-I, as judged from the results with the intermediate gel technique. No such component has been reported to occur in normal plasma, but analogous observations on cholestatic plasma were earlier reported by other authors using immunoelectrophoresis [25, 29].

On zonal ultracentrifugation the cholestatic HDL patterns had two characteristic features: (i) a decrease of HDL₂ and HDL₃ with a concomitant disappearance of the normally distinct demarcations of these two subclasses, and (ii) the appearance of a small but distinct component of relatively low density, called cholestatic HDL₁-C [6].

This component showed a very peculiar protein and a lipid composition distinctly different from that of normal HDL. In addition, the fractions corresponding to HDL₂ and HDL₃ were different from their normal counterparts. The most striking feature was the presence of an apoprotein most reasonably identical to the

arginine-rich protein or apo E [31] as judged from its molecular weight and the immunoreaction against anti-apo E. This component dominated the protein part of HDL₁-C, but was also present in heavier HDL fractions. Furthermore, the main part of the cholesterol in HDL₁-C was unesterified. The occurrence of apo E in HDL₁-C is consistent with Utermann's finding of this apoprotein in cholestatic HDL₂ isolated by differential flotation [31].

No conclusive explanation can so far be offered for the drastic alterations of HDL in biliary obstruction but the similarities between the cholestatic HDL patterns and the pattern found in patients with familial LCAT deficiency are striking. Plasma from such patients contains very little α -lipoprotein as judged by lipoprotein electrophoresis and also the crossed immunoelectrophoretic patterns against anti-HDL, closely resembles those of cholestatic plasma [8]. Decreased cholesterol esterifying ability of cholestatic plasma has been demonstrated in cases of long-standing cholestasis [12, 32, Arnesjö *et al.*, unpublished observations]. Electron microscopic images of cholestatic plasma have also revealed the presence of particles with the same shape and dimensions as those described in LCAT deficiency [16].

The occurrence of a HDL component with the low density of 1.08 g/cm³ (HDL₁-C) in cholestatic plasma is consistent with the finding of a HDL fraction with apparently large particle size, which behaved like HDL₁-C in crossed immunoelectrophoresis. Similar gel filtration results have been obtained earlier in cholestasis [5] and in LCAT deficiency [23].

The dominance of an apoprotein, most likely identical to apo E in the protein part of HDL₁-C is interesting in view of the finding by Norum *et al.* [24] of this component in HDL from patients with LCAT deficiency and a lack of this apoprotein in the VLDL from the same patients. It is possible that HDL₁-C is identical to 'nascent' HDL isolated by Hamilton & Kayden [15]. These authors found that perfusates from rat liver in the presence of an inhibitor of LCAT activity contained HDL particles with abundant apo E. Such particles are assumed to undergo a rapid transformation under the influence of LCAT with a concomitant loss of apo E. The transformation might be inhibited in cholestasis either by a decrease of the LCAT concentration or possibly by an interference of bile salts with

the LCAT activity in the way proposed by Hamilton & Kayden [15].

The HDL₁-C readily obtained by zonal centrifugation of plasma from patients with long-standing obstructive jaundice might serve as a suitable material for further studies of the formation and metabolic fate of human HDL.

ACKNOWLEDGMENT

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ISOLATION AND PARTIAL CHARACTERIZATION OF LIPOPROTEIN E FROM PATIENTS WITH LONG-STANDING BILIARY OBSTRUCTION

B. Danielsson¹, R. Ekman², B.G. Johansson², P. Nilsson-Ehle²,
and B.G. Petersson³

Departments of Biochemistry¹, Clinical Chemistry², and Surgery³,
University of Lund, Lund, Sweden

ABSTRACT

The abnormal HDL fraction (HDL₁-C) occurring in obstructive jaundice has been separated by zonal ultracentrifugation and further studied by electron microscopy and immunochemical techniques. HDL₁-C, which is characterized by high concentrations of apo E, phospholipids and unesterified cholesterol, was found to consist exclusively of disc-shaped particles with a diameter of 15-25 nm. The ratio of apo E/apo A-I in HDL₁-C was around 4.5; however, the distributions of the two apoproteins in the HDL₁ density region did not coincide. Studies with immunosorbent chromatography and crossed immunoelectrophoresis with an intermediate gel further demonstrated a heterogeneity within the HDL₁-C fraction and gave strong evidence for the presence of a population of lipoprotein particles containing apo E as only protein constituent (LpE). The appearance of HDL₁-C (and LpE) in biliary obstruction seems to be related to the low endogenous cholesterol esterifying ability in these patients (5-20% of normal). No bile salts, which have been suggested to inhibit LCAT in biliary obstruction, could be detected in the HDL₁-C fraction.

INTRODUCTION

Pronounced changes of high density lipoproteins (HDL) are a common finding in patients with intra- or extrahepatic biliary obstruction (1-5). Studies in patients with acute alcohol hepatitis (6) or with long-standing extrahepatic cholestasis (7-9) reveal changes of HDL which are similar to those found in familial lecithin:cholesterol acyltransferase (LCAT) deficiency. The isolation of an abnormal HDL₁ subclass from cholestatic plasma by zonal ultracentrifugation was recently described (9). This lipo-

protein fraction, called HDL₁-C, has an unusual protein and lipid composition, characterized by large proportions of apoprotein E (arginine-rich protein) and a high ratio of free cholesterol/cholesteryl ester (9). Similar data were reported by Hamilton et al. (10) for HDL isolated from rat liver perfusates in the presence of an inhibitor of LCAT.

The present work was undertaken in order to extend the characterization of the abnormal HDL₁ lipoprotein from cholestatic plasma. The results demonstrate a heterogeneity of the lipoprotein particles of the HDL₁-C fraction, and suggest that part of the particles represents newly synthesized lipoprotein E particles (primary LpE particles, cf. 11).

MATERIALS AND METHODS

Source of plasma

Blood samples were drawn in tubes containing Na₂EDTA (1 mg/ml) after an overnight fast from patients with long-standing obstructive jaundice due to pancreatic carcinoma (3 patients) or alcoholic liver disease (2 patients). Plasma was separated within two hours after collection of the blood and the ultracentrifugation separation was started within six hours.

Isolation of cholestatic HDL₁

HDL₁-C was isolated from 30 ml plasma samples by zonal ultracentrifugation as described earlier (12). The HDL₁-C fraction was dialyzed for 3 hrs against distilled water at 8°C (Spectrapore tubing, M.W. cut-off 3500, Spectrum Medical Industries, Inc., Los Angeles) and concentrated by dialysis against a 40% (w/v) solution of polyethylene glycol (mean M.W. 20 000) to a protein concentration of 0.5-1 g/l.

Preparation of antigens and antisera

Isolation of apo E from HDL₁-C mainly followed the instructions given by Curry et al., who used very low density lipoproteins (VLDL) as starting material (13). The HDL₁-C fraction was dialysed against 0.01 M ammonium bicarbonate buffer (pH 8.0) and lyophilized. The powder was extracted five times with chloroform:methanol (2:1, v/v) and twice with diethyl ether at 8°C. The delipidized HDL₁-C was solubilized in 6 M guanidine hydrochloride, containing 0.01 M ammonium bicarbonate and 0.05% Na₂EDTA. Apo E was isolated by chromatography on a Sepharose

CL-6B column (1.6 x 80 cm) equilibrated with 6 M guanidine hydrochloride. Fractions containing only apo E, as judged by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), were pooled, dialyzed extensively against distilled water, and lyophilized.

Amino acid composition of apo E was determined in a Durrum D-500 amino acid analyzer. The results were in accordance with earlier reports (14-16).

Antisera to apo E were raised in rabbits following the immunization schedule described earlier (17). Preparation of antisera to human lipoproteins A-I, A-II, B and C-I has been described previously (17,18). Immunoglobulin preparations from antisera mainly followed the instructions given by Steinbuch and Audran (19).

Preparation of Sepharose/anti-A-I immunosorbent

To 2.0 g of cyanogen-bromide (CNBr) activated Sepharose 4 B (Pharmacia Fine Chemicals, Uppsala, Sweden) was added 20 mg of purified human apo A-I (18) dissolved in 0.1 M NaHCO_3 buffer (pH 8.3) containing 0.5 M NaCl. The protein-gel suspension was mixed in an end-over-end mixer (Rotamix) overnight at $+8^\circ\text{C}$. A ratio of 2 mg of protein/ml activated Sepharose was utilized, and a coupling efficiency of $>95\%$ was determined from protein analyses. 15 ml of anti-A-I immunoglobulin fraction was passed through a Sepharose-A-I affinity column (0.9 x 7.5 cm) which was followed by 20 ml of a 0.01 M Tris-HCl, pH 7.4, 0.5 M NaCl buffer. The column was equilibrated with 3 M NaSCN, and after about 18 hrs the dissociated anti-apo-A-I was eluted with the same buffer. Approximately 50% of the total bound material was recovered after this treatment as judged from protein determinations. The purified antibodies obtained were used to prepare an immunosorbent to apo A-I in the same fashion, using 12 mg of purified antibodies and 2 mg CNBr-activated Sepharose. The coupling efficiency was $>95\%$.

Immunochemical methods

Electroimmuno assay (EIA) was performed as described by Laurell (20). Plasma samples were delipidated with diisopropylether:n-butanol 60:40 (v/v) as described by Cham and Knowles (21) prior to EIA. Crossed immunoelectrophoresis with one or several intermediate antibody-containing gels was carried out according to Axelsen (22). In this procedure the first step involves a regular electrophoresis of the antigen ($\text{HDL}_1\text{-C}$) in agarose gel. In the second step a new electrophoresis is performed,

perpendicularly to the first one, allowing the antigen(s) to migrate through two or more gels containing different antibodies (e.g. against apo A-I, apo A-II, apo C-I and apo E). Lipoprotein particles may precipitate or pass the intermediate gels, depending on their apoprotein composition; only particles lacking a specific apoprotein will be able to pass through an intermediate gel containing the corresponding antibody.

Chemical methods

The lipid composition of lipoproteins was determined as described previously (9).

The fatty acid composition of cholesteryl esters was determined by gas liquid chromatography as described by Åkesson et al. (23). Fractionated phosphatides were estimated using the method described by Bartlett (24). Bile acids were determined following the method of Brunsgaard (25). Protein concentrations were determined by the method of Lowry et al. (26). Polyacrylamide gel electrophoresis was carried out according to King and Laemmli (27). The proportions of apo A-I, apo A-II and apo E in lipoproteins were estimated by the procedure described by Friedberg and Reynolds (28) involving treatment of apoprotein mixtures with fluorescamine followed by SDS-PAGE and subsequent elution and fluorescence measurement of the separated apoproteins.

Endogenous cholesterol esterifying ability was determined as described by Stokke and Norum (29).

Electron microscopy

Lipoprotein samples (0.5-1 g/l) were dialyzed overnight against 0.1 M ammonium acetate (pH 7.4) and then mixed with an equal volume of 2% (w/v) sodium phosphotungstic acid (pH 7.4). The samples were applied on carbon-coated copper grids and examined under a Philips EM 300 electron microscope.

RESULTS

All patients had greatly reduced endogenous cholesterol esterifying ability (mean 0.45%, range 0.21-0.90%). Control sera, assayed on the same occasion, had values between 3.5 and 5%, which is in the same order of magnitude as the data reported by Stokke and Norum (29). Plasma concentrations of apo A-I, as measured by EIA using delipidated plasma,

were also very low (not measurable in one patient, and 12, 10, 7 and 7% of the apo A-I concentration of a plasma pool from healthy volunteers).

A representative pattern of the HDL obtained by zonal ultracentrifugation of plasma from patients with long-standing biliary obstruction is shown in Fig. 1 a. The lipoprotein fraction HDL₁-C used for further studies was recovered within the density interval 1.08-1.10 kg/l. Electron microscopy of this fraction revealed only stacked disc-like particles with average dimensions of 4 x 20 nm (Fig. 2). No spherical particles corresponding to normal HDL₂ or HDL₃ were seen.

The distributions of apo A-I and apo E are shown in Fig. 1 b. Apo A had a bimodal distribution with one peak in the density region around 1.19 kg/l and the other one in the HDL₁ region. Apo E also showed two major peaks, one in HDL₁ and the other one in the low density lipoproteins (LDL). No apo B was detected by EIA in the HDL region including HDL₁. It should be noted that the distributions of apo A and apo E in the HDL₁ did not coincide.

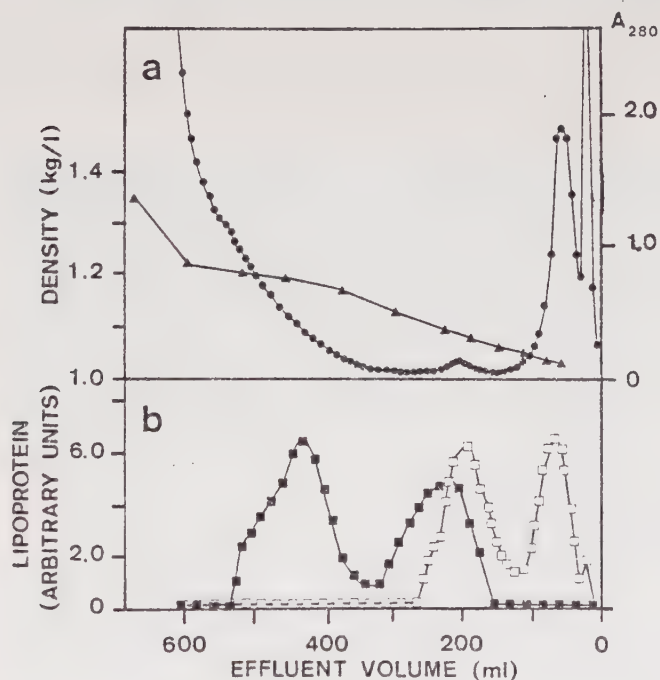


Fig. 1. Zonal ultracentrifugation in a continuous sodium bromide gradient of 30 ml plasma from a patient with biliary obstruction. The rotor was run for 20 hrs at 44 000 rpm at 15°C. a) ●—●, absorbance at 280 nm; ▲—▲, density at 15°C. b) electroimmuno assay against apo A-I (■—■) and apo E (□—□).



Fig. 2. Electron microscopy of HDL₁-C. Negative stain; x 188 000.

Apo E was the main apoprotein in HDL₁ as judged from SDS-PAGE (Fig. 3). The molar ratio apo E:apo A-I was around 4.5:1 in HDL₁-C from two patients when determined with SDS-PAGE of fluorescent labeled apoproteins. These calculations were based on the assumption of a molecular weight of 36 000 for apo E.

Immunochemical techniques were used to demonstrate the heterogeneity of the HDL₁-C particles with regard to apoprotein composition. Crossed immunoelectrophoresis with an intermediate gel containing anti-A-I, anti-A-II or anti-C-I and an upper gel with anti-E showed in all experiments a distinct precipitate in the anti-E-containing gel. Fig. 4 illustrates the results obtained when a mixture of anti-A-I, anti-A-II and anti-C-I is used in the intermediate gel. The area delineated by the immunoprecipitate against anti-E was reduced to about 50 per cent of the corresponding area in a control plate with no antibody in the intermediate gel. These results indicate that a considerable part of the particles contained virtually only apo E as protein constituent.

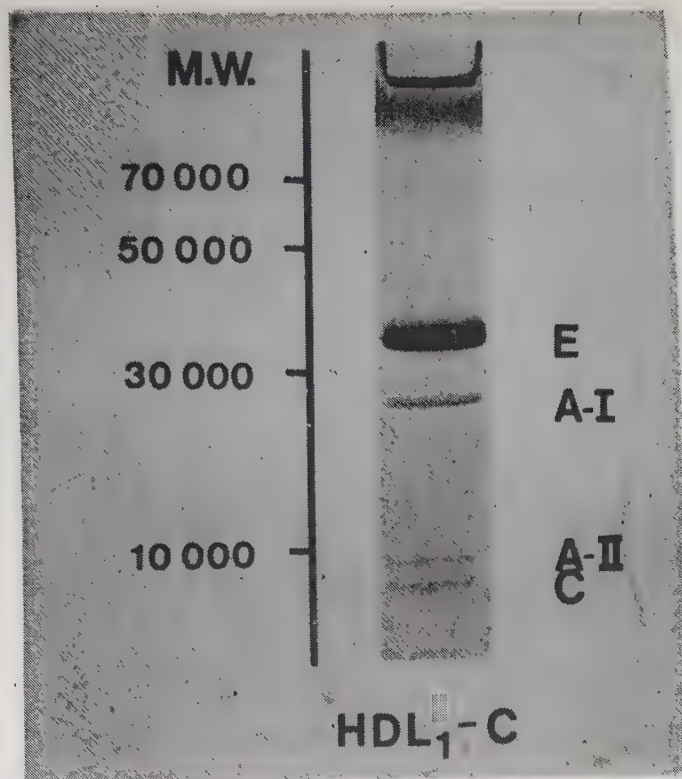


Fig. 3. Electrophoresis of apoprotein from isolated HDL₁-C in 12% polyacrylamide containing 0.1% SDS. The sample was reduced with 2-mercaptoethanol. The approximate molecular weight scale was deduced from the positions of nine standard proteins.

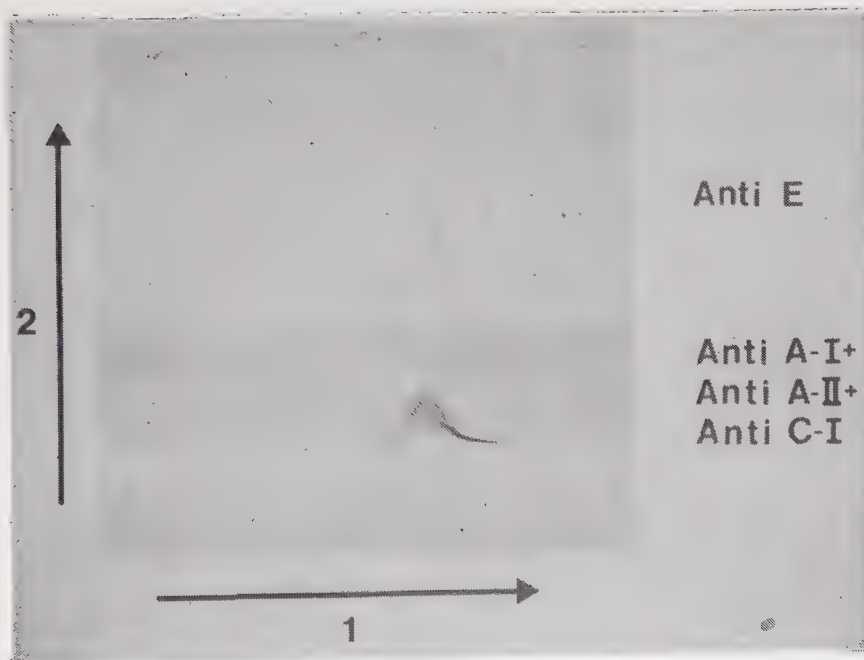


Fig. 4. Crossed immunoelectrophoresis of HDL₁-C. The intermediate gel contained anti-A-I, anti-A-II, and anti-C-I, and the reference gel anti-E. Arrow 1 indicates the anodal direction in the first step, and arrow 2 the anodal direction in the second step.

Immunsorbent chromatography on anti-A-I-Sepharose columns revealed that about 70% of the apo E in HDL₁-C was not bound, as judged by apo E determinations with EIA before and after chromatography. The SDS-PAGE patterns of the unretained and retained fractions are shown in Fig. 5.

We have earlier demonstrated that about 70% of the lipids of HDL₁-C is phospholipid (9). The distribution of phospholipid species in HDL₁-C is summarized in Table 1, showing a predominance of phosphatidyl choline. The lysophosphatidyl choline amounted to less than 4.5%. The cholesterol in HDL₁-C is present mainly in unesterified form (9). The fatty acid composition of the minor cholesteryl ester fraction in two of the patients is given in Table 2. The most abundant fatty acid was palmitic acid, whereas the linoleic acid constituted a much lower proportion of the esterified fatty acid than in normal HDL₂. No bile acids were detected in any of the five HDL₁-C fractions studied (the sensitivity of the method allowed detection of an amount corresponding to 0.8% of the sample analyzed).

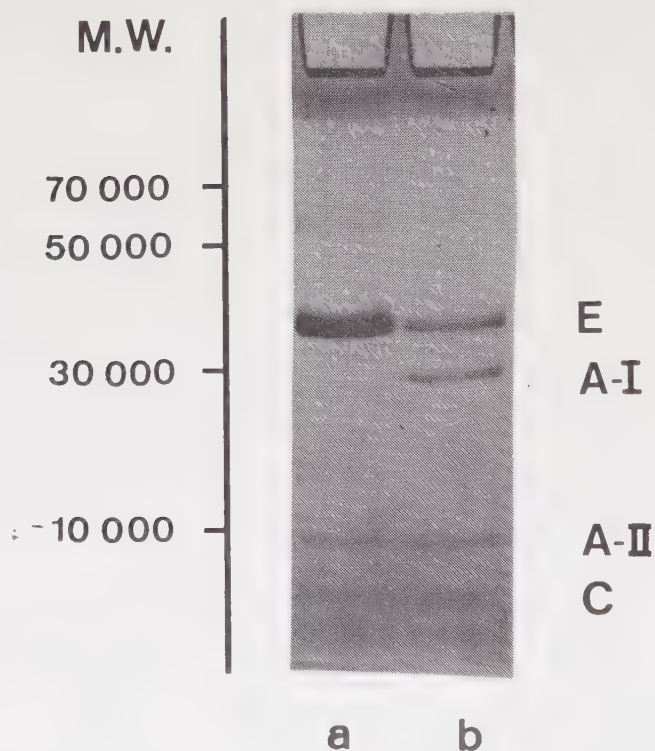


Fig. 5. Electrophoresis in 12% polyacrylamide containing 0.1% SDS of subfractions of HDL₁-C. HDL₁-C was fractionated by immunsorbent chromatography on anti-A-I-Sepharose. a) represents the unretained and b) the retained fraction. Samples were reduced by 2-mercaptoethanol.

TABLE 1

Main phospholipid species of the HDL₁-C fraction. Data are given as per cent of total lipid phosphorus (% by weight)

Phospholipid	Mean (n = 4)	Range
Phosphatidyl choline	77	73-80
Lysophosphatidyl choline	2.5	1.8-4.5
Sphingomyelin	12	9-14

TABLE 2

Fatty acid composition of cholesteryl esters in the HDL₁-C fractions separated by zonal ultracentrifugation from two patients (A and B) with biliary obstruction due to pancreatic carcinoma. Data from normal HDL₂ are shown for comparison

Fatty acid	HDL ₁ -C (% of weight)		HDL ₂ (% of weight)
	A	B	
C 16 : 0	19	38	12
C 16 : 1	5	12	2
C 18 : 0	6	5	<1
C 18 : 1	56	26	19
C 18 : 2	14	18	66

DISCUSSION

The isolation by zonal ultracentrifugation of an abnormal HDL fraction, cholestatic HDL or HDL₁-C, from patients with obstructive jaundice has recently been reported (9). The fraction was characterized by a predominance of apo E and of phospholipids and unesterified cholesterol. It was suggested that the appearance of this abnormal HDL was related to a decreased LCAT activity in patients with obstructive jaundice.

In the present study we show by electron microscopy that the HDL₁-C fraction consists of disc-shaped particles exclusively, a finding which is in agreement with earlier reports of the appearance of HDL in cholestasis (7, 8). Similar abnormalities of HDL have also been described in familial LCAT deficiency (7, 30, 31). All patients had a drastic decrease in LCAT activity. Although a content of lysophosphatidyl choline comparable with that of normal HDL₂ was found, the low content of linoleic acid (18:2) in the minor cholesteryl ester fraction indicates that the esterification of cholesterol in HDL₁-C is largely dependent on enzymes other than LCAT. The cause of the reduced LCAT activity in obstructive jaundice is not known. Bile salts have been suggested to play a role for a reduction of LCAT activity by displacing the cofactor apo A-I from the surface of the HDL particles (31). In this study, however, no measurable amounts of bile salts were present in HDL₁-C. Other more likely possibilities are reductions of the concentration of the enzyme per se or of its cofactor, apo A-I. The morphological similarities of HDL₁-C and HDL in familial LCAT deficiency may implicate that a reduction of enzyme concentration may play a role, but on the other hand total plasma concentrations of apo A-I were extremely low, and at least a fraction of the heterogeneous HDL₁-C subclass seemed to contain no apo A-I. The fact that the HDL₁-C fraction is a good substrate for LCAT (7; R. Ekman, unpublished observations) may favour the former possibility.

The finding of a predominance of apo E in the cholestatic HDL₁ fraction is consistent with observations by Hamilton et al. (10) who reported large amounts of apo E in HDL obtained by perfusion of rat liver in the presence of ditionitrobenzoic acid (DTNB), an inhibitor of LCAT activity. Further studies have shown that apo E and apo A have considerably different secretion rates from rat liver (32), which might indicate the formation and secretion of different "primary" (11) lipoprotein particles. In the present study the observation of a different distribution of apo E and apo A-I in the HDL₁ region indicates a heterogeneity of the apo-protein composition of the HDL₁-C particles. This heterogeneity was

further studied by immunochemical methods.

By immunosorbent chromatography on immobilized anti-A-I a lipoprotein containing 70% of the apo E in HDL₁-C was found to pass the column unretained. This fraction did not contain measurable amounts of apo A-I, indicating that apo A-I and apo E were at least partly present in different particles. Since the unretained lipoprotein fraction besides apo E also contained small amounts of apo A-II and apo C, crossed immunoelectrophoresis with anti-E and an intermediate gel containing a mixture of apo A-I, apo A-II and apo C-I was used to further elucidate the distribution of apoproteins in lipoprotein particles. This arrangement prevents lipoprotein particles containing apo A-I, apo A-II and apo C from migrating into the upper anti-E-containing gel. The distinct precipitate in the gel containing anti-E indicates the presence of lipoprotein particles containing only apo E as protein constituent. It should be noted, however, that this interpretation is valid only under the assumption that no dissociation of apolipoproteins from HDL₁-C occurs during electrophoresis, and that all apo C (C-I, C-II and C-III) are present in the same particles (as suggested by the results of Kostner and Alaupovic (33)), since an antiserum against only C-I was used.

The present results are interesting in relation to the lipoprotein family concept introduced by Alaupovic and coworkers (34), which is also the main basis for the classification of plasma lipoproteins in terms of "primary" and "secondary" complexes suggested by Osborne and Brewer (11). In accordance with these concepts we suggest that part of the disc-shaped particles in the HDL₁-C fraction constitutes a lipoprotein E family or primary lipoprotein E particle. Evidence for the existence of a lipoprotein E family in normal human HDL has been presented by Curry et al. (13), and recently Ragland et al. (35) suggested that human lipoprotein E, like rat lipoprotein E (10), may be synthesized by the liver and excreted as HDL. No detailed studies on the composition of human plasma lipoprotein E are yet available.

The mechanism behind the appearance in cholestatic plasma of the postulated LpE particles is not known, but it is reasonable to assume that it is related to the reduced LCAT activity in plasma. Forte et al. (7) could demonstrate a change in HDL from disc-shaped to spherical particles upon addition of LCAT to plasma from patients with familial LCAT deficiency or biliary obstruction. Similarly, incubation of HDL₁-C with purified LCAT for 24 hours results in a considerable increase in esterified cholesterol and a partial change from disc-formed to spherical par-

ticles (Ekman et al., unpublished results), but it is still unclear if all lipoprotein particles in HDL₁ can serve as a substrate for LCAT, or if this reaction is limited to particles containing apo A-I.

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Changes in plasma high density lipoproteins in chronic male alcoholics during and after abuse

B. DANIELSSON, R. EKMAN,* G. FEX,† B. G. JOHANSSON,*
H. KRISTENSSON,‡ P. NILSSON-EHLE* & J. WADSTEIN‡

Institute of Biochemistry, University of Lund, Department of Clinical Chemistry, University Hospital, Lund,* Department of Clinical Chemistry† and The Alcohol Clinic,‡ Malmö General Hospital, Malmö, Sweden

Danielsson, B., Ekman, R., Fex, G., Johansson, B.G., Kristensson, H., Nilsson-Ehle, P. & Wadstein, J. Changes in plasma high density lipoproteins in chronic male alcoholics during and after abuse. *Scand. J. clin. Lab. Invest.* 38, 000-000, 1978.

Alterations in plasma high density lipoproteins (HDL) were studied in thirty-eight male chronic alcoholics. Twenty-four (63%) of the patients had increased HDL protein (measured immunochemically) and twenty-five (66%) had increased HDL cholesterol (determined after polyanion precipitation of very low density lipoproteins (VLDL) and low density lipoproteins (LDL)). A statistically significant correlation was found between HDL protein and HDL cholesterol ($r=0.39$). γ -Glutamyltransferase (GT) was elevated in eighteen (47%) of the alcoholics. No significant correlations were found between GT and HDL protein or HDL cholesterol. The increase in HDL, as studied by rate zonal ultracentrifugation, was heterogeneous with changes in the HDL₂ as well as HDL₃ sub-fractions. It is suggested that determination of HDL total cholesterol, in combination with GT, may represent a valuable and sensitive test for detection of alcoholism.

Key-words: alcoholic intoxication; high density lipoproteins; lipoprotein cholesterol; serum enzymes; zonal ultracentrifugation

Dr R. Ekman, Department of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden

Disturbances in plasma lipoprotein metabolism is a common finding in alcoholism. Plasma triglyceride concentration is often elevated, sometimes to very high levels [5, 15, 21, 26, 35], as a result of increases in the concentrations of very low density lipoproteins (VLDL) and chylomicrons. That there is also an increase in plasma high density lipoproteins (HDL) [13] in alcoholism is less well known. Recently, however, Johansson & Medhus [14] showed that about

80% of chronic male alcoholics had an increased concentration of HDL as measured immunochemically.

The present investigation was undertaken to confirm this observation and to determine if other quantitative or qualitative changes in HDL were present. We have determined HDL immunochemically, total and unesterified cholesterol in HDL and studied the subclasses of HDL using density gradient centrifugation in a zonal rotor. These determinations have been

made on samples obtained immediately after an acute alcohol abuse and during a recovery phase of 2–3 weeks.

MATERIAL AND METHODS

Patients

Thirty-eight male chronic alcoholics, selected according to Jellinek's classification [11], were studied. Their age was 24–59 years. All were intoxicated on admission with plasma ethanol concentrations between 24 and 62 mmol/l. Their last bout had lasted for more than 4 weeks and most of them were in poor physical condition. Their diet before admission was not controlled.

In a group of eight patients it was possible to obtain serial blood samples to elucidate the time-course of the HDL alterations after cessation of ethanol intake. These patients were kept in hospital for 1 week and then controlled at regular intervals for 3 weeks. In this group additional studies were performed in order to describe more in detail the nature of the HDL changes (i.e. determinations of HDL total and esterified cholesterol, HDL ultracentrifugation pattern).

Clomethiazol (500–750 mg three to four times daily), alone or together with prometazin (15 mg three to four times daily), was given to the eight patients during the first week. From the second week the patients were given dixyrazin (10–25 mg three times daily) and nitrazepam (5–10 mg in the evening). Disulfiram was given to all patients starting a week after admission.

Twelve apparently healthy men aged 48 years served as controls. Venous blood samples were taken in tubes containing Na₂-EDTA after the patients had fasted overnight. Samples designated 'on admission' or not further specified were drawn 12–24 h after the patients had arrived at the ward. All samples were kept at +4°C and analysed within 48 h.

Methods

Antisera against lipoproteins were raised in rabbits. Anti-HDL serum was produced by immunization with HDL isolated by ultracentrifugation in the density interval 1.063–1.210 kg/l [10]. Anti-low density lipoprotein

(LDL) serum was obtained by immunization with LDL (1.006–1.063 kg/l) [10]. The antisera against HDL and LDL were specific in crossed immunoelectrophoresis [8].

HDL protein was determined by electro-immuno assay [19] and the results were expressed as per cent of the value of pooled plasma from healthy subjects (fifty-eight men aged 48 years). This pooled plasma was stored in aliquots at –20°C. This method had a precision corresponding to a coefficient of variation of 5%.

Isolation of HDL of individual plasma samples for cholesterol determination was performed by selective precipitation of LDL and VLDL [4] as follows: to 1 ml of plasma was added 10 µl of 10% (w/v) dextran-sulphate 500 (AB Pharmacia, Uppsala, Sweden) and 50 µl of 1 mol/l MgCl₂. The tubes were shaken, allowed to stand at +4°C overnight and then centrifuged at 2000 rpm for 10 min. The analyses were performed on the clear supernatant. No LDL protein was found in the supernatant as tested with electroimmuno assay [19] and radial immunodiffusion [22] with anti-LDL protein. The recovery of HDL protein in the supernatant was better than 90% as tested with electroimmuno assay and radial immunodiffusion with anti-HDL serum. The coefficient of variation of the HDL cholesterol determination was 8%.

Cholesterol was determined enzymatically [29]. Unesterified cholesterol was determined with the same method by excluding cholesterol esterase from the reaction mixture. Triglyceride was determined enzymatically [34] using glycerol as standard.

The separation of HDL into subfractions mainly followed the procedure described by Danielsson, Ekman & Petersson [6] using a Beckman L 2–65B ultracentrifuge with the following modifications: the zonal rotor (Ti-14) was loaded from the edge at 3000 rpm with 50 ml buffer (0.01 mol/l Tris-HCl and 1 mmol/l EDTA, pH 7.4) serving as an overlay followed by 550 ml NaBr-gradient linear with respect to volume and with a density range of 1.000–1.290 kg/l. The sample (20 ml) adjusted to 1.295 kg/l in density with solid NaBr was then injected at the periphery of the rotor followed by 30 ml of a NaBr solution density 1.300 kg/l as cushion. The centrifugation was carried out at 46,000 rpm (max RCF 158,000 g) at +15°C

for 20 h. The rotor content was displaced from the rotor at 3000 rpm with a NaBr solution of density 1.400 kg/l. The absorbance at 280 nm was continuously registered and 15 ml fractions were collected. The density of the fractions was analysed by weighing 10 ml aliquots. Appropriate fractions were pooled and their apoprotein composition monitored by sodium dodecylsulphate-polyacrylamide gel electrophoresis [17].

Ethanol was determined fluorimetrically [7]. Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) activity was determined with a Vitatron Akes reaction rate analyser using reagents from AB Kabi, Stockholm, Sweden. γ -Glutamyltransferase (GT) activity was determined with γ -glutamyl-*p*-nitroanilide as substrate [27].

Statistical calculations

SD represents the standard deviation of a sample and *n* the number of subjects. Precision is expressed as relative SD (coefficient of variation). Reference values for laboratory determinations represent mean $\pm$ 2 SD for an apparently healthy population. Significance of differences between groups was analysed with Student's *t* test.

RESULTS

On admission twenty-seven and twenty-five of the thirty-eight patients in the present study had increased activities of ALAT and of ASAT, respectively. Eighteen of the patients had increased activity of GT (Table I).

Six of the thirty-eight patients and two of the

twelve controls had increased plasma triglyceride levels (>2.2 mmol/l) and one of the patients and one of the controls had increased plasma cholesterol level (>8.3 mmol/l) during the study period (Table I).

The HDL concentrations obtained by electro-immuno assay and HDL cholesterol values are presented in Fig. 1. The mean HDL protein concentration of the patients was $139 \pm 30\%$ (mean $\pm$ SD, *n*=38), which was significantly ($P<0.0001$) higher than that of the controls ($96 \pm 14\%$, *n*=12). Similarly, the HDL cholesterol concentration of the patient group was 2.1 ± 0.7 mmol/l, *n*=38, which was increased ($P<0.0001$) compared to the control group (1.1 ± 0.3 mmol/l, *n*=12). 63% and 66% of the patients had values exceeding mean of control group + 2 SD for the HDL protein and HDL cholesterol determinations, respectively. Values for the eight subjects volunteering for further studies seemed to be randomly distributed among the patients (Fig. 1).

There was a good correlation between plasma HDL protein and HDL cholesterol concentration among the controls ($r=0.74$; $P<0.01$) as well as among the patients ($r=0.39$; $0.01<P<0.05$) (Fig. 1). It is evident, however, that there is a substantial variation between the patients, some of which demonstrate a normal HDL protein concentration but increased HDL cholesterol concentration, and *vice versa*.

No correlation between HDL protein or HDL cholesterol and serum GT activity was found ($r=0.04$ and 0.09 , respectively; *n*=38). Nor could correlations between HDL cholesterol or HDL protein and ASAT or ALAT activities be demonstrated. Plasma triglyceride concentrations, however, were correlated to serum GT activities ($r=0.50$; $P<0.01$; *n*=38),

TABLE I. Plasma aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and γ -glutamyl-transpeptidase (GT) activities and plasma triglyceride (TG) and cholesterol concentrations in controls and in alcoholics 12–24 h after admission to hospital

	ASAT (μ kat/l)	ALAT (μ kat/l)	GT (μ kat/l)	TG (mmol/l)	Cholesterol (mmol/l)
Reference values	<0.7	<0.7	0.24–0.74	0.3–2.2	3.2–7.7
Mean (range) of controls (<i>n</i> =12)	nd	nd	0.40 (0.19–0.78)	1.76 (0.40–3.25)	6.69 (4.12–8.69)
Mean (range) of patients (<i>n</i> =38)	1.44 (0.33–6.05)	1.17 (0.24–5.28)	1.67 (0.22–9.40)	1.53 (0.62–5.75)	5.20 (3.29–8.96)

nd, Not determined.

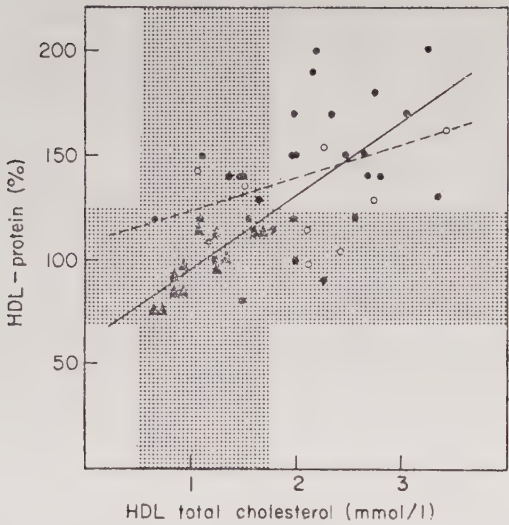


FIG. 1. Linear regressions for values of plasma HDL protein and HDL cholesterol concentrations in controls (indicated by triangles) and patients (indicated by circles). The shaded area indicates mean ± 2 SD for the control group. Regression equations $y=37.2x+54.7$ (controls, solid line) and $y=16.1x+105$ (patients, dotted line). Correlation coefficients were 0.91 and 0.38 for controls and patients, respectively. Open circles indicate values for the eight patients participating in the time-course study. Values for HDL protein concentration (measured immunochemically) are expressed relative to those of pooled plasma from fifty-eight healthy males.

whereas no correlation between plasma triglyceride levels and ASAT or ALAT activities was found. No statistically significant correlations between plasma triglyceride concentrations and HDL protein or HDL cholesterol could be demonstrated ($r=-0.28$ and -0.07 , respectively; $n=38$).

Table II shows a comparison of the frequency

TABLE II. Frequency of increased plasma GT activities, HDL protein, and HDL cholesterol concentrations in thirty-eight alcoholics 12–24 h after admission to hospital

	No.	%
Within reference values in all variables	2	(5%)
Increased GT	18	(47%)
Increased HDL protein	24	(63%)
Increased HDL cholesterol	25	(66%)
Increased GT or HDL protein	30	(79%)
Increased GT or HDL cholesterol	31	(82%)
Increased HDL protein or HDL cholesterol	32	(84%)
Increased GT, HDL protein or HDL cholesterol	36	(95%)

of increased levels of GT activities (which has been suggested as a laboratory test for alcoholism) and HDL protein and cholesterol concentrations.

Eight of the thirty-eight patients participated in further studies of the composition of the increased HDL lipoprotein fraction, which was monitored serially up to four weeks after admission. The total as well as the unesterified cholesterol concentrations in HDL were significantly ($P<0.05$) higher than normal in seven

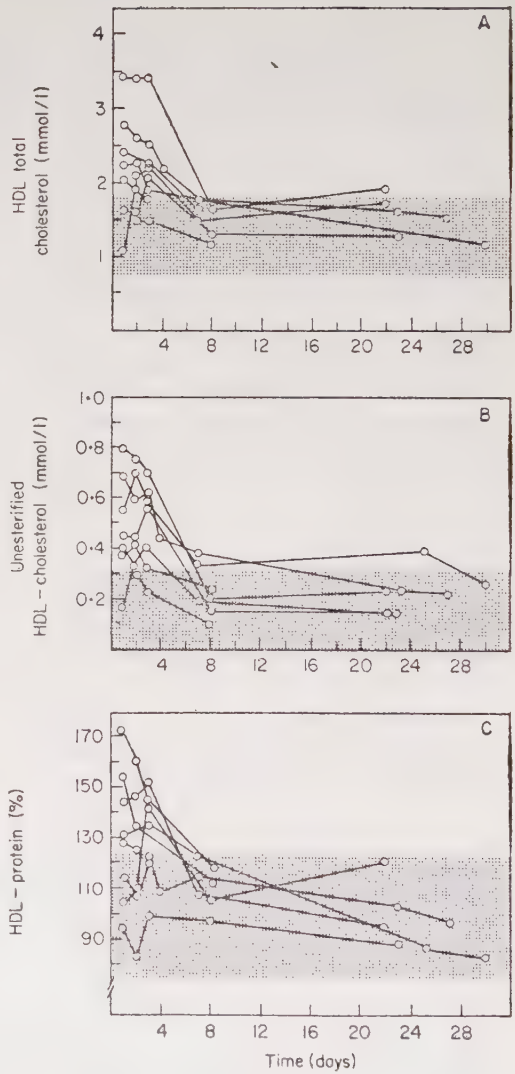


FIG. 2. Time-course for changes of the levels of HDL constituents after admission to hospital. The shaded area represents mean ± 2 SD of the control group. (A) Total cholesterol in HDL, (B) unesterified HDL cholesterol, and (C) immunochemically determined HDL protein.

of the eight patients on one or more of the three days after admission (Fig. 2a, b). HDL free cholesterol was increased in both absolute and relative terms (cholesterol/cholesterol ester ratio elevated), but expressed in absolute values, the

increase in cholesterol ester was greater than that in free cholesterol. All patients but two had a concomitant increase in HDL protein measured immunochemically during one or more of the first 3 days (Fig. 2c). HDL concentrations returned to values within the reference range within a week, as judged from all three variables.

The characteristics of the HDL increase was further studied by separation of subfractions of HDL by rate zonal ultracentrifugation. This was performed on serial samples from the eight patients participating in the time-course study described above.

The distribution of plasma lipoproteins separated by zonal ultracentrifugation is shown in Fig. 3. Peak I represents a mixture of VLDL and LDL well separated from HDL₂ (peak II) and HDL₃ (peak III). All patients showed abnormal distribution patterns of HDL subclasses as judged from the absorbance at 280 nm. Five patients showed a moderate increase of a somewhat broad and asymmetrical HDL₃ fraction with a shoulder of a relatively small HDL₂ component (Fig. 3b). In the other three patients, in contrast, a four- to five-fold increase of a symmetrically distributed HDL₂ fraction was seen as the main alteration (Fig. 3c). The apoprotein composition in the partially separated subfractions was not analysed in detail, but the distribution of the main apoprotein constituents (AI and AII) did not show any gross abnormalities as judged by visual inspection of sodium dodecylsulphate-polyacrylamide gel electrophoresis patterns.

The qualitative alterations in HDL were normalized in parallel with HDL cholesterol and protein during a period of 1 week. After this period of time the patients with an increase in predominantly HDL₂ all exhibited normal proportions of HDL subfractions with an HDL₃/HDL₂ ratio >1 (Fig. 3c, d).

DISCUSSION

In a previous study by Johansson & Medhus [14] it was shown that about 80% of male alcoholics had elevated concentrations of HDL protein as measured immunochemically. The present study confirms and extends these data, demonstrating increased concentrations of HDL protein in about 65% of a group of thirty-eight

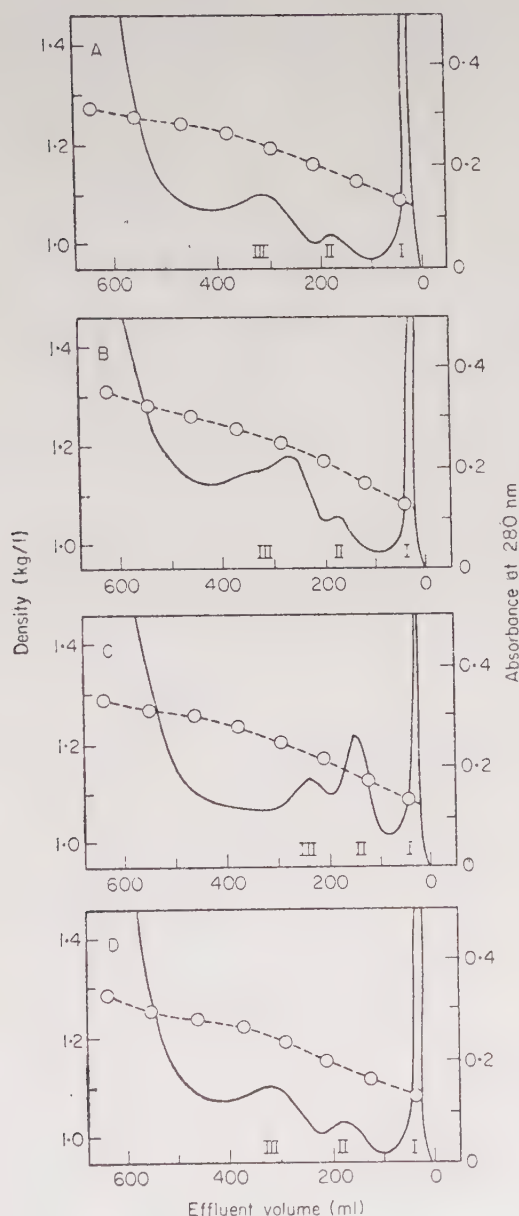


FIG. 3. Zonal ultracentrifugation patterns of plasma from (A) a normal male, (B) and (C) two patients immediately after admission, (D) the latter patient 1 week after admission. The dotted line represents density and the full line absorbance at 280 nm. Peak I represents VLDL and LDL, peak II HDL₂, and peak III HDL₃.

chronic alcoholics and elevated levels of HDL cholesterol in about the same percentage of the patients (but not in the identical individuals, see below). The increased HDL cholesterol concentrations could be ascribed to an increase in free as well as esterified cholesterol.

In the control and in the patient group, HDL protein and HDL cholesterol concentrations were significantly correlated. There was, however, especially among the patients, a certain variation, and several subjects had normal values in one of these determinations but increased values in the other (Fig. 1), implicating varying proportions between the protein and lipid moieties and a varying composition of the increased HDL fraction in alcoholism. This notion is consistent with the ultracentrifugal profiles, which showed no uniform pattern but revealed changes in the HDL₂ as well as in the HDL₃ area. It is interesting to note that the patients showing an increase mainly in the less dense HDL₂ fraction had a much more pronounced increase in HDL cholesterol than HDL protein, the latter variable being well within the normal range (Fig. 1).

The reason for the increased HDL concentration in the alcoholics is unknown at present. Apart from alcohol, a possible malnutrition of the patients on admission and/or the treatment given to them after admission might have influenced the HDL values. The latter was probably unimportant in this study since the HDL values were increased before any treatment was given. Reliable information on the diet during the period preceding the admission was impossible to obtain. Thus the question of malnutrition in the patients cannot be definitely settled. However, in a group of healthy volunteers [2, 3] alcohol, when added to the normal diet, increased HDL protein concentration, and cessation of alcohol administration led to a decrease. Thus it seems safe to conclude that the increase in HDL is related to alcohol consumption. The plasma protein pattern, studied in eight patients by agarose gel electrophoresis [12] in combination with specific immunochemical quantitation, was normal in five cases and showed signs of liver involvement (high α_1 -antitrypsin level, normal orosomucoid level, and low haptoglobin level) in three of the patients [16]. Plasma ceruloplasmin concentrations were within normal limits in all subjects, implying that the HDL increase in these patients was not

mediated via oestrogen [20]. Oestrogen influence tends to increase plasma HDL levels [1, 9, 32] and gives an ultracentrifugal pattern similar to that shown in Fig. 3c [28].

A number of reports have shown elevation of GT activities to occur in alcoholism [18, 24, 25, 30, 31] and determination of plasma GT activity has been suggested as a tool to detect alcoholism in a subjectively healthy population [31]. However, this indicator has some drawbacks. Other studies have reported that one third to a quarter of the patients with high plasma GT activities are not alcoholics, and a proportion of alcoholics, varying between 10 and 50% in different studies, do not have elevated plasma GT activities [14, 31].

There is thus a need for additional simple laboratory tests to detect alcoholism. Recently it was reported that an increased α -amino-*n*-butyric acid/leucine ratio in plasma could be used to detect alcohol abuse [33]. Such analyses are, however, complicated and will probably be available in only a few clinical laboratories.

The elevated HDL concentrations seem to offer a possible complement to plasma GT determinations in the diagnosis of alcoholism. Since the two variables were not correlated they probably reflect different effects of ethanol or ethanol intake. This may also be true for the HDL protein and HDL cholesterol concentrations which, although significantly correlated, showed a considerable variation (Fig. 1). There seemed to be no apparent advantage to measure HDL unesterified cholesterol compared to HDL total cholesterol (Fig. 2).

In the present study, comprising thirty-eight chronic alcoholics, determinations of plasma GT activity revealed abnormally high values in about 50% of the subjects. A combination of plasma GT determinations and plasma HDL protein or cholesterol measurements demonstrated one or two abnormal values in about 80% of the group, whereas essentially all patients had elevated levels of one or more of the three variables GT activity, HDL cholesterol and HDL protein. Determinations of HDL total cholesterol, which is a technically simple procedure, and/or HDL protein, thus seem to increase the possibility to obtain a laboratory diagnosis of alcoholism. A further evaluation of these methods will require additional studies, especially with regard to falsely positive results and to the application in other

populations, such as subjects with other drinking habits [23] and in women.

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ISOLATION OF A HIGH DENSITY LIPOPROTEIN WITH HIGH CONTENTS OF ARGININE-RICH APOPROTEIN (apoE) FROM RAT PLASMA

Bengt DANIELSSON[†], Rolf EKMAN, Bengt G. JOHANSSON, Peter NILSSON-EHLE
and Bengt G. PETERSSON*

*Departments of Clinical Chemistry, [†]Biochemistry and *Surgery, University of Lund, Lund, Sweden*

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1. Introduction

Although the rat has been extensively used as an animal model for studies on the plasma lipoprotein metabolism, the protein components of the rat lipoprotein classes have not until recently been explored in greater detail. It has been shown [1,2] that the protein part of HDL which is the major lipoprotein density class of the rat is more complex than that of human HDL. The presence of C-proteins and of monomeric apoA-II in rat HDL was demonstrated [3]. Using SDS-polyacrylamide gel electrophoresis, a major apoprotein with mol. wt 27 000 was shown [4] to be analogous to human apoprotein A-I. Two additional components not present in normal human HDL were found in rat HDL. One of these, which had app. mol. wt 46 000, has no known human analogue. The other one which had approx. mol. wt 35 000, is homologous with human arginine-rich protein [5] also called apoE [6].

The presence of apoE in normal human HDL is under debate; however, it has been demonstrated that HDL from patients with familial LCAT deficiency or longstanding biliary obstruction contains considerable amounts of apoE [6,7]. In a current study of

lipoprotein patterns of rats with induced hepatobiliary damage we have found that the normal rat contained an HDL species with an abundance of apoE but little or no apoA-I. The isolation by zonal ultracentrifugation and partial characterization of this lipoprotein will be described here.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (250–300 g) were fed a standard laboratory diet ad libitum for at least 4 days. The animals were fasted for 16 h before aortic puncture under light ether anaesthesia. The blood was collected in the presence of Na₂EDTA (1 mg/ml) and plasma separated within 1 h. Plasma from 10–15 rats were pooled and ultracentrifuged within 5 h.

2.2. Zonal ultracentrifugation

Lipoprotein fractionation was performed in a Beckman-Spinco ultracentrifuge model L2-65 equipped with a Ti-14 rotor. A density gradient of NaBr (550 ml, 1.00–1.29 kg/l, linear to rotor volume) was used for the fractionation of lipoproteins from 20–30 ml rat plasma. An overlay of 30 ml 0.01 M Tris-HCl (pH 7.4) containing 1 mM Na₂EDTA ($d = 1.0$ kg/l) and a cushion of NaBr solution ($d = 1.30$ kg/l) were used to fill the rotor completely. Ultracentrifugation was carried out at 15°C for 15 h at 46 000 rev/min (max RCF = 158 000 $\times g$). The rotor content was collected in 7.5 ml fractions and A_{280} determined for each fraction.

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoprotein; LCAT, lecithin cholesterol acyl transferase; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoC, C apolipoproteins; apoE, arginine-rich apolipoprotein; SDS, sodium dodecylsulphate

Address correspondence to: R. Ekman

Pooled fractions were extensively dialyzed (Spectrapore tubing, mol. wt cut-off 3500, Spectrum Medical Industries, Los Angeles) to remove NaBr and concentrated by dialysis against a 40% (w/v) solution of polyethylene glycol (mean mol. wt 20 000) to a protein concentration of 0.5–1 g/l.

2.3. Electroimmunoassay

Electroimmunoassay was performed as in [8] with an antiserum to dog β -lipoprotein [9] showing distinct immunoreactivity against rat LDL.

2.4. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in 8 M urea after delipidation with tetramethyl urea [10] or in SDS [11]. Samples were reduced with 2-mercaptoethanol before electrophoresis in SDS.

2.5. Lipid analyses

Lipids were extracted with chloroform/methanol, 2:1 (v/v). The main lipid classes were separated by thin-layer chromatography on silica gel with petroleum ether/ethyl ether/glacial acetic acid (90:10:1) as mobile phase. The lipids were localized by iodine vapour and eluted from the scraped areas by chloroform/methanol, 1:1.

Triglycerides were determined with an enzymatic method [12] with reagents from Boehringer Mannheim. Cholesterol and cholesterol esters were determined enzymatically [13]. Phospholipids were determined by analysis of phosphorus in the lipid extract [14].

2.6. Electron microscopy

Lipoprotein samples (0.5–1 g/l) were dialyzed overnight against 0.1 M ammonium acetate and then mixed with equal vol. 2% (w/v) sodium phosphotungstate, pH 7.4. The samples were immediately applied to carbon-coated copper grids and examined under a Philips EM 300 electron microscope.

3. Results

Zonal ultracentrifugation under the conditions described gave a separation of the main lipoprotein density classes and the bulk of plasma proteins as indicated in fig.1. Peak I consisted of VLDL followed

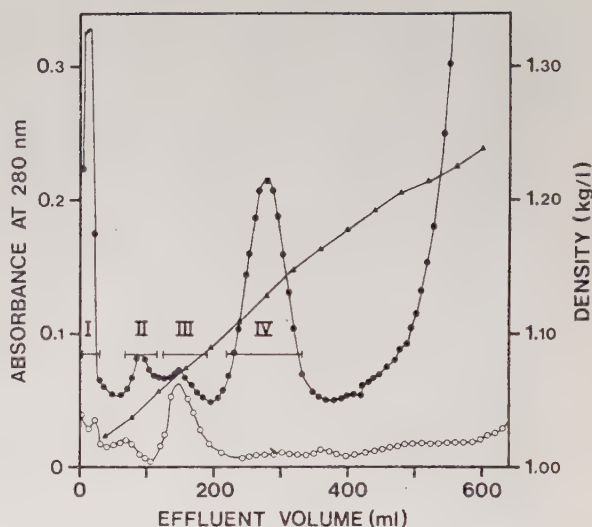


Fig.1. Lipoprotein profile of rat plasma separated by zonal ultracentrifugation. Plasma, 25 ml applied in a discontinuous sodium bromide gradient. For experimental details see section 2. (●—●) A_{280} ; (▲—▲) density at 20°C; (○—○) A_{280} fraction III recentrifuged under the same conditions. Fractions were pooled as indicated by the horizontal bars.

by an LDL fraction (peak II) at a density of 1.04 kg/l. The LDL fraction was small in comparison with the HDL fraction (peak IV). The HDL peak, recovered at 1.13 kg/l, was homogeneous as judged from the density distribution, and well separated from the bulk of the plasma proteins. In contrast to human plasma, rat plasma contained a lipoprotein fraction with a density between LDL and the main HDL (peak III, fig.1). This lipoprotein fraction had an apparent density of 1.08 kg/l and was not totally separated from the other lipoprotein classes.

Recentrifugation of fraction III was performed under the same conditions as the original centrifugation of rat plasma. As seen in fig.1 most material was recovered in a symmetrical peak in the density region around 1.08 kg/l. In electron microscopy, this fraction was shown to consist of spherical particles with an average diameter of about 14 nm (fig.2).

The apoprotein composition of the zonal ultracentrifugation fractions was studied with SDS–polyacrylamide gel electrophoresis. The HDL patterns were characterized by three main components with app. mol. wt 28 000, 36 000, 44 000 (fig.3d) and small amounts of low mol. wt (8000–12 000)

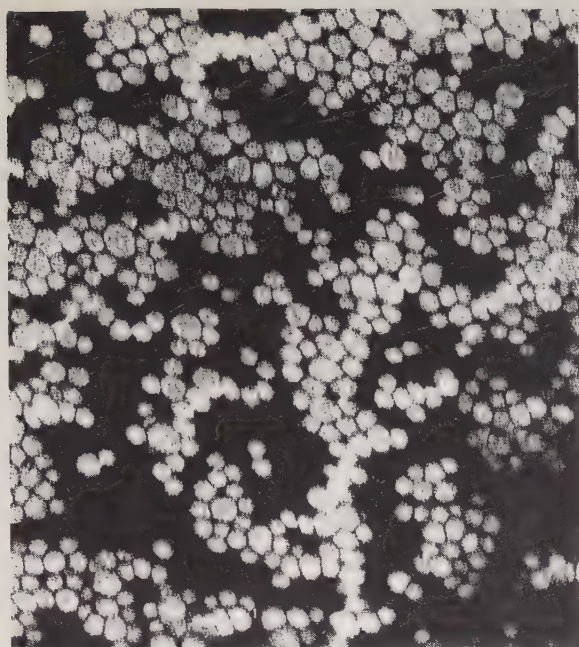


Fig.2. Negatively-stained HDL particles isolated at an average density of 1.08 kg/l ($\times 166\,440$).

material. The component with mol. wt 28 000 was the most prominent one and constituted more than half of the protein part of HDL.

The material in the fraction collected between LDL and HDL (fraction III in fig.1) had an apoprotein composition quite different from that of HDL. As seen in fig.3c the component with mol. wt 35 000 was very prominent, while the component with mol. wt 28 000 was present in much smaller amounts. Small amounts of low molecular weight components were also present as in HDL. The presence of an apoprotein analogous to dog B-protein in fractions I and II was established by the distinct immunoprecipitates

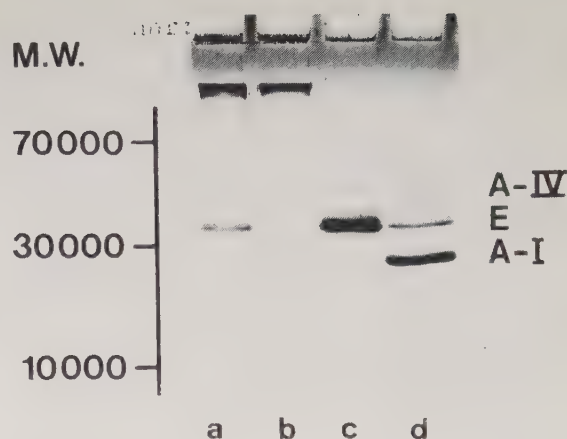


Fig.3. SDS-polyacrylamide gel electrophoresis of lipoproteins separated by zonal ultracentrifugation (cf. fig.1). Separation performed in 12% polyacrylamide. The approximate molecular weight scale was deduced from the position of 9 standard proteins. (a) Fraction I (VLDL); (b) Fraction II (LDL); (c) Fraction III ('HDL₁'); (d) Fraction IV (HDL).

obtained on electroimmunoassay against an antiserum to dog B-lipoprotein. Such material was present in, at most, trace amounts in fractions III and IV.

The total protein concentration of fraction III was 25–35% (w/w) compared to 44% (w/w) for HDL (fraction IV). The lipid composition of rat HDL and the intermediate fraction (fraction III) is shown in table 1. No striking differences were observed.

4. Discussion

Rat plasma lipoproteins were prepared by differential ultracentrifugation [1–4]. The gradient centrifugation in zonal rotors makes it possible to study, on a

Table 1
Lipid composition of the intermediate fraction (III) and HDL (IV) (% w/w)

		Triglyceride	Cholesteryl esters	Free cholesterol	Phospholipids
Fraction III	Exp. 1	6.3	38.9	12.6	42.0
	Exp. 2	4.7	35.5	9.4	50.4
HDL (Fraction IV)		4.9	38.0	4.8	52.3

preparative scale, the density distribution of lipoproteins without the fixed and predetermined density intervals used in the differential centrifugation technique. The present results show that the main lipoprotein density classes of rat plasma may be isolated in a single overnight run, as previously shown for other species, i.e., human [15], pig [16] and dog [17]. The present results confirm that rat LDL constitutes a comparatively small part of the total lipoprotein, which is dominated by HDL [18]. The rat HDL did not separate into subclasses as human and canine HDL [15,17], but appeared homogeneous in its density distribution as porcine HDL [16].

The apoprotein composition of the HDL isolated by zonal ultracentrifugation was in accordance with [4] where apoA-I, arginine-rich protein (apoE) and a component with app. mol. wt 46 000 (A-IV) were found.

The fraction between LDL and HDL (fraction III) contained only small amounts of apoA-I. The major apoprotein in this fraction was most probably identical with apoE. This apoprotein composition is of interest in relation to [19] where separate distributions of apoE and apoA-I on Sepharose gel filtration of rat plasma were found, consistent with the appearance of a lipoprotein E in the present zonal ultracentrifugal experiments. The rat lipoprotein described herein has no known counterpart in the normal human lipoprotein spectrum. A human lipoprotein with a similar apparent density of 1.08 kg/l has been isolated from patients with longstanding cholestasis [15] and is most probably also present in plasma from individuals with alcoholic liver disease [20]. The human abnormal lipoprotein, tentatively called HDL₁-C [15] also contains apoE as the major apoprotein, but the unique lipid composition of HDL₁-C is quite different from the rat lipoprotein which has approximately the same lipid composition as normal rat HDL with a major part of its cholesterol esterified. This fact also excludes the possibility that the lipoprotein described here is closely related to 'nascent' rat HDL described [21]. In addition, electron microscopic images obtained by us reveal a 'normal' spherical appearance of the lipoprotein particles rather than the disc-like rouleaux forming particles demonstrated [21].

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